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CLINICAL AND EXPERIMENTAL

A STUDY OF THE ABSORPTION AND UTILIZATION OF AN IRON CHELATE IN IRON-DEFICIENT PATIENTS

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CINCINNATI, OHIO

THE absorption of inorganic iron salts from the gastrointestinal tract is incomplete in normal subjects and in patients with hypochromic anemia. Tracer studies with isotopic iron have shown that 79 to 98 per cent of the administered dose of iron is recoverable in the stools of normal subjects and 29 to 76 per cent in the stools of patients with hypochromic anemia.[1] For this reason iron in a form more readily absorbable would be advantageous in treating iron deficiency anemia. It has been reported that iron in the form of ferric potassium chelate is more effective than ferrous sulfate in treating iron deficiency chlorosis in grapefruit trees.[2] Iron tightly bound in a chelate such as ethylenediaminetetra-acetic acid (Versene), is readily soluble in water, nonionized, and fails to give the usual chemical reactions of ionized iron. These distinctive properties might determine the absorption of an iron chelate and the "iron acceptor" mechanism[3] of the gastrointestinal tract might be bypassed, permitting a greater absorption of iron.

To test this hypothesis the absorption and utilization of an iron chelate, ferric sodium Versenate* was measured in three ways: (1) Increases in reticulocytes and hemoglobin induced by suboptimal doses of ferrous sulfate and ferric sodium Versenate were compared in each of five patients with hypochromic anemia. (2) The rate of hemoglobin regeneration per day was calculated in patients with hypochromic anemia on full therapeutic doses of ferric sodium Versenate. (3) Ferric sodium Versenate and ferrous sulfate containing isotopic iron were administered to normal subjects and patients with hypochromic anemia. Stool and urine specimens were studied for the presence of radioactive material.

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MATERIAL AND METHODS

Five patients with hypochromic anemia were given suboptimal doses of ferrous sulfate for a period of two weeks, followed by an equivalent dose of ferric sodium Versenate for a period of two weeks (15 to 30 mg. elemental iron per day). No patient was bleeding during the period of study. Hemoglobin was measured as oxyhemoglobin and erythrocytes were enumerated by standard counting techniques. Reticulocyte counts were performed by the wet method using a modified Dameshek's stain. These determinations were made every other day during the period of study.

Three patients with hypochromic anemia were given potentially therapeutic doses of ferric sodium Versenate (100 mg. elemental iron three times a day) until they had normal hemoglobin levels. The studies were terminated in the third case (J. B.) by an acute bleeding episode. Hemoglobin was measured at weekly intervals as oxyhemoglobin and the rate of hemoglobin regeneration per day was calculated.

Ferric sodium Versenate containing 20 μc of radioactive iron and from 20 to 30 mg. of elemental iron was administered orally to one normal adult man and to one adult man recovering from iron deficiency anemia whose iron stores had not been completely replenished by iron therapy. After the oral dose of ferric sodium Versenate, twenty-four-hour collections of stool were obtained for three to five days, and urine for three days. Random red blood cell samples were obtained from one to nine days and the radioactivity contained in each specimen was measured. Similar determinations were carried out on two normal men and one normal woman after administering an oral dose of ferrous sulfate containing 20 μc of radioactive iron and from 20 to 30 mg. of elemental iron.

The measurement of radioactivity of stool and urine specimens was carried out in the following manner. The twenty-four-hour stool specimens were collected in wide-mouthed jars. The entire twenty-four-hour specimen was transferred to a Waring Blendor with careful rinsing of the jar with distilled water. A sufficient amount of water was added to permit blending of the stool to a thick slurry. Blending was carried out for 30 minutes to permit even distribution of particulate matter. The slurry was transferred to volumetric flasks followed by distilled water rinses of the Blendor jar. Amyl alcohol was added to the slurry to prevent foaming. Distilled water was used to bring the slurry to a convenient volume, usually 500 to 1,000 ml. Three milliliter aliquots of the slurry were counted in a "well type" scintillation counter. A radioactive iron standard was prepared at the same time as the test dose of radioactive iron. All radioactive specimens were counted at the same time as the standard specimen to eliminate interpolations for decay. Table I indicates the accuracy of this method in nine recovery experiments. Vary-

TABLE I. EFFICIENCY OF RECOVERY OF RADIOIRON ADDED TO FECES

SAMPLE	CHEMICAL FORM OF RADIOIRON ADDED	VOLUME OF SLURRY (ML.)	RADIOIRON ADDED (COUNTS/SEC.)	RADIOIRON FOUND (COUNTS/SEC.)	RECOVERY RADIOIRON (PER CENT)
1	Ferric Versenate	500	8,010	7,763	96.3
2	Ferric Versenate	1000	3,054	2,725	89.3
3	Ferric Versenate	1000	8,144	7,355	90.3
4	Iron globulin complex	500	1,996	1,700	85.2
5	Iron globulin complex	500	4,991	4,683	93.8
6	Iron globulin complex	500	9,982	8,900	89.2
7	Iron globulin complex	500	6,717	6,033	89.9
8	Iron globulin complex	500	6,717	5,850	87.1
9	Iron globulin complex	500	6,717	5,966	88.8

ing amounts of radioactive ferric sodium Versenate and iron globulin complex were added to stool specimens. The average recovery was 89.9 per cent of the added radioactivity.

Twenty-four-hour urine specimens were collected and the volume measured. Three milliliter aliquots of the urine were counted directly in the "well type" scintillation counter, at the same time as the standard specimen of radioactive iron.

RESULTS

Only slight to moderate reticulocytosis occurred in the five patients with hypochromic anemia who were given suboptimal doses of ferrous sulfate for two weeks, followed by an equivalent dose of ferric sodium Versenate. There was no significant change in hemoglobin levels and no significant difference in the reticulocyte responses to either of these two iron compounds. The results are tabulated in Table II.

The results on daily hemoglobin regeneration in the three patients who received potentially therapeutic doses of ferric sodium, Versenate are tabulated in Table III. The average rates of hemoglobin regeneration over the first 56 days of treatment in Case 1 (D. E.) and in Case 2 (J. J.) were 0.085 and 0.102 Gm. hemoglobin per 100 ml. of blood per day, respectively. During the first 35 days of treatment in Case 3 (J. B.) the rate was 0.220 Gm. per 100 ml. per day. The rate of hemoglobin formation with ferric sodium Versenate is no greater than with inorganic salts of iron, which, reports indicate, may induce an average hemoglobin rise of 0.17 Gm. per 100 ml. of blood per day and in many cases of 0.25 Gm. per 100 ml. of blood per day for the first two to four weeks of treatment.[4]

The results of the studies with ferric sodium Versenate and ferrous sulfate containing radioactive iron are tabulated in Table IV. In the control group receiving ferrous sulfate, 61 to 91 per cent of the radioactive iron was recovered in the stool. In one case (M. H.) the first twenty-four-hour urine specimen showed the presence of definite radioactive material but at such a low level that quantitation was not possible. The red blood cell samples in two cases (M. J. and M. S.) showed significant amounts of radioactivity in samples obtained as early as two days after the dose of radioactive iron was administered. Greater amounts were found in specimens obtained seven and eight days after the oral dose. No attempt was made to estimate the per cent of the administered dose of radioactive iron contained in the hemoglobin as no blood volume studies were made.

Eighty-four per cent of the administered dose of radioactive iron was recovered in the stool of the normal subject who received ferric sodium Versenate. No urinary output of radioactive material could be detected. In the iron-deficient subject who received radioactive ferric sodium Versenate, 47 per cent of the radioactive material was recovered in the stool and a trace of radioactive material was found in the first twenty-four-hour urine specimen. Random red blood cell specimens showed the presence of radioactive material twenty-four hours after the oral dose of iron and greater amounts were found in specimens obtained on the third and sixth days after the oral dose.

TABLE II. RETICULOCYTE AND HEMOGLOBIN RESPONSE TO SUBOPTIMAL DOSES OF FERROUS SULFATE AND FERRIC SODIUM VERSENATE

PT.	INITIAL HEMATOLOGICAL DATA						ELEMENTAL IRON DOSE PER DAY MG.	FERROUS SULFATE TREATMENT PERIOD				FERRIC SODIUM VERSENATE TREATMENT PERIOD			
								RETICULOCYTES		HEMOGLOBIN		RETICULOCYTES		HEMOGLOBIN	
	RBC (MILLIONS)	HGB (6/N/100 ML.)	HCT (%)	MCV (CU. MICRONS)	MCHC (%)	MCH (γγ)		INITIAL	MAXIMUM	INITIAL	MAXIMUM	INITIAL	MAXIMUM	INITIAL	MAXIMUM
L. A.	4.97	6.7				13	15	1.3	2.8	6.7	6.6	1.4	2.0	6.9	6.6
D. W.	5.08	8.0				16	15	1.4	5.0	8.0	7.7	1.3	2.4	7.8	8.0
S. H.	2.68	4.6	17	63	27	17	30	0.9	14.1	4.6	6.7	1.4	12.1	6.3	8.6
C. H.	3.55	8.0	29	81	27	22	20	1.4	4.8	8.0	9.1	3.1	3.5	9.1	9.0
C. C.	3.39	8.75	31	81	25	28	20	5.6	4.0	8.75	9.7	2.9	4.2	9.7	11.0

TABLE III. HEMOGLOBIN REGENERATION WITH THERAPEUTIC DOSES OF FERRIC SODIUM VERSENATE

PT.	INITIAL HEMATOLOGICAL DATA						NUMBER OF DAYS TREATED	TOTAL DOSE OF ELEMENTAL IRON (GM.)	DAY OF THERAPY	HGB (GM./100 ML.)	RATE OF HGB REGENERATION (GM./100 ML./DAY)
	RBC (MILLIONS)	HGB (GM./100 ML.)	HCT (%)	MCV (CU. MICRONS)	MCHC (%)	MCH (γγ)					
D. E.	5.92	10.0	40	68	25	17	59	17.7	14	10.1	0.071
									21	11.45	0.069
									35	12.1	0.057
									49	13.7	0.088
									56	14.9	0.085
									77	15.5	0.070
J. J.	3.53	8.6				24.3	56	16.8	28	11.2	0.093
									49	11.8	0.065
									56	14.3	0.102
J. B.	3.05	4.9	20	65	24	16	35	10.5	8	6.2	0.163
									13	8.2	0.254
									21	11.5	0.314
									35	12.6	0.220

TABLE IV. RADIOACTIVE IRON CONTENT OF STOOL AND URINE AFTER ORAL ADMINISTRATION OF ISOTOPIC FERROUS SULFATE AND FERRIC SODIUM VERSENATE

PT.	HEMATOLOGIC DATA						MEDICATION	TOTAL TEST DOSE RADIO IRON (COUNTS/SEC.)	RADIO IRON RECOVERED	
	RBC (MILLIONS)	HGB (GM./100 ML.)	HCT (%)	MCV (CU. MICRONS)	MCHC (%)	MCH (γγ)			STOOL (PER CENT)	URINE (PER CENT)
M. H.	5.16	17.2	51	98	33	33	$FeSO_4$	135,900	61	0.3
M. J.	4.05	14.0	40	98	34	35	$FeSO_4$	146,595	69	0.0
M. S.	4.00	13.5	41	102	33	32	$FeSO_4$	135,287	91	0.0
C. S.	4.82	15.3	49	101	31	31	Fe Versene	163,500	84	0.0
J. B.	4.50	12.6	40	88	28	31	Fe Versene	174,500	47	0.39

DISCUSSION

Iron absorption from the gastrointestinal tract is incomplete in normal subjects. Larger amounts are absorbed by patients who are iron deficient. The mechanism of the body which controls iron balance is not fully understood but there is evidence that an "iron acceptor" compound[3] exists in the intestinal mucosa, which is capable of regulating the absorption of iron from the gastrointestinal tract. If an iron compound could be found which would bypass this mechanism, greater amounts of iron might be made available to the body for hemoglobin production and for replenishing iron stores. Since ferric sodium Versenate is a tightly bound chelate of iron, the suggestion has been made that the chemical properties of the molecule might be determined by the Versene rather than the iron, and absorption might not depend on the "iron acceptor" compound[3] of the gastrointestinal mucosa. If absorbed, however, ferric sodium Versenate might not relinguish its iron because of the strong bind of iron to Versene. If this situation existed, a high urinary excretion of ferric sodium Versenate would be anticipated because Versene is not metabolized. Intravenous injection of heavy metal chelate compounds is followed by a high urinary excretion of these compounds.[5, 6] However, when isotopic ferric sodium Versenate was administered to a normal subject the majority of the radioactive iron was recovered from the stool. The quantity was similar to the amount of radioactive iron recovered in the stool of control subjects receiving isotopic ferrous sulfate and was in the same range as reported by Moore in a normal group receiving isotopic ferrous chloride.[1]

The iron-deficient patient receiving isotopic ferric sodium Versenate had less radioactive iron remaining in his stool than the normal subjects, but no less radioactive iron than Moore's iron-deficient group receiving isotopic ferrous chloride.[1] The radioactive iron that was absorbed appeared in the circulating red blood cells indicating that the iron was available for hemoglobin formation. Negligible radioactivity appeared in the urine indicating that iron, as the Versenate, was not absorbed and excreted unchanged by the kidneys. These observations are consistent with the recent report that no more than 5 per cent of C^{14} labeled calcium Versenate is absorbed after oral administration to human beings.[6]

These studies suggest that the iron chelate is split within the gastrointestinal tract releasing elemental iron for absorption by the usual mechanism. The stools of patients taking large doses of ferric sodium Versenate became greenish black in color indicating the formation of an iron sulfide. This is a further indication that at least some of the ferric sodium Versenate is split as the Versene complex will not react with sulfides.

The final proof of the absorption and availability of an iron compound lies in its ability to produce reticulocytosis and to increase hemoglobin in iron-deficient patients. The clinical observations in iron-deficient patients who received ferric sodium Versenate indicate that ferric sodium Versenate is capable of producing reticulocytosis and hemoglobin rise, but that these responses

are no greater than usually occur when equivalent doses of ferrous sulfate are administered. This is in keeping with the study using isotopic ferric sodium Versenate which suggests splitting of the complex in the gastrointestinal tract and absorption of the iron in ionic form.

SUMMARY AND CONCLUSIONS

1. Ferric sodium Versenate has been administered to normal control subjects and to iron-deficient patients. Determinations have been made of the extent to which it is absorbed and becomes available to the body for hemoglobin formation.

2. Studies with isotopic labeled ferric sodium Versenate demonstrate that iron from this complex is utilized in hemoglobin formation but that absorption from the gastrointestinal tract is no greater than with ferrous sulfate. There is no significant urinary excretion of radioactive material.

3. Reticulocyte response to ferric sodium Versenate in suboptimal dosage occurs but is of no greater magnitude than to the equivalent dose of ferrous sulfate.

4. The rate of hemoglobin regeneration per day on full dosage of ferric sodium Versenate is no greater than on the equivalent dose of ferrous sulfate.

5. The observations suggest that some of the iron chelate is split in the gastrointestinal tract into ionized iron, and that this iron is absorbed by the usual gastrointestinal mechanism which maintains normal iron balance.

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SIMULTANEOUS ADMINISTRATION OF CORTICAL STEROIDS AND ACTH: THE EFFECT ON ADRENAL WEIGHT AND CHOLESTEROL CONCENTRATION

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SOME time after the advent of long-term cortisone therapy, it became evident that termination of such treatment was often followed by symptoms indicating adrenal cortical insufficiency. Stebbins[1] reported atrophy of the adrenal cortex following prolonged cortisone therapy in animals and O'Donnell and associates[2] and Fraser and colleagues[3] noted the same phenomena in man. Stebbins[4] has also shown that the atrophied adrenals of the hypophysectomized rat are not further diminished in weight by prolonged treatment with large doses of cortisone.

Cortisone-induced adrenal atrophy has raised important clinical problems as well. In 1950, Forsham and co-workers[5] reported that the cortisone-suppressed adrenal cortex failed to show clinical response to ACTH stimulation for as long as ten days after termination of therapy. Similar findings were published by McIntosh and Holmes,[6] and Wilkins and associates.[7]

Although it was found that adrenal atrophy was reversible and that increased urinary excretion of ketosteroids was observed following an intercurrent infection even in a patient who failed to show response to a diagnostic test with ACTH, it does not indicate, however, that the chronically cortisone-treated patient is capable of withstanding a major stress. An illustrative case of fatal surgical shock in just such a patient has been described by Fraser, Preuss, and Bigford.[3]

It seemed important, therefore, to determine whether or not it was possible to induce adrenal regeneration after atrophy had occurred, even though cortisone was continued, and more important still, whether the simultaneous administration of ACTH and cortisone would prevent adrenal atrophy. Because of clinical interest in compound F (hydrocortisone) and compound B (corticosterone) which developed at the time this work was in progress, the experiment was expanded to include these materials.

MATERIALS AND METHODS

Male white rats weighing approximately 100 grams were obtained from the Holtzmann Rolfsmeyer Company. After one week in our laboratory, they were subjected to hypophysectomy according to the technique of Munson and associates.* Only those animals which did not show appreciable change in weight (more than ± 7 grams) during the following week were used in the subsequent experiments. Control studies were carried

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out in intact animals and in 12, 22, and 28 days after hypophysectomy, and seven groups were given various treatments according to the following schedules, treatment beginning on the seventh day after hypophysectomy:

Group I: Cortisone actetate, 5 mg. of the aqueous suspension, was injected once daily for 15 days beginning on the seventh day after operation.

Group II: Hydrocortisone (compound F), 5 mg. of the aqueous suspension, was injected once daily for 15 days.

Group III: Corticosterone (compound B), 5 mg. of the aqueous suspension, was injected daily for 15 days.

Group IV: ACTH, 1 mg. of an aqueous solution (Armour Lot 212-103), was injected three times daily for 15 days.

Group V: Cortisone acetate, 5 mg. daily, and ACTH, 1 mg. three times daily, were injected according to the same schedule.

Group VI: Hydrocortisone, 5 mg. daily, and ACTH, 1 mg. three times daily, were injected as previously mentioned.

Group VII: Corticosterone, 5 mg. daily, plus ACTH, 1 mg. three times daily, were injected as previously described.

Immediately after the last dose of steroid and/or ACTH, all animals were given 0.5 ml. of dilute India ink solution into the tail vein (Higgins American India Ink diluted 1:10 with saline). The cachectic state of many animals at this time made the procedure difficult and their tails were first immersed in hot water for 1 to 2 minutes to bring about venous distention. Untreated controls were injected with India ink at the same interval after hypophysectomy as the treated groups. Exactly twenty-four hours after ink injection, the animals were anesthetized with ether, weighed, and killed by exsanguination. The pituitary fossa was examined in all animals to determine whether hypophysectomy was complete and only the completed ones were included in the data. Liver and spleen were removed, enclosed in parafilm, weighed, and portions taken for histologic study and for photometric determination of India ink uptake. The techniques and results of tissue India ink uptake studies will be reported elsewhere. The adrenals were dissected out, weighed, and the cholesterol content determined according to a slightly modified Schoenheimer-Sperry method using duplicate determinations on each filtrate and including standards and recoveries with each group of filtrates.

RESULTS

Results of four control and seven tested groups are summarized in Table I.

Control Studies.—The adrenal weight of intact animals of this strain averaged 19.6 ± 2.7 mg. per 100 grams body weight. Twelve days after hypophysectomy, the adrenal weight had decreased to 10.2 ± 1.3 mg. per 100 grams. (There was no further change over the time interval studied, for the average of 10.5 ± 0.8 mg. at 22 days, and 12.4 ± 2.0 mg. per 100 grams at 28 days is not a statistically significant change from the average weight 12 days postoperatively.) This is evidence that hypophysectomy was complete, for the lack of adrenal weight gain indicates an absence of endogenous ACTH production. The adrenal cholesterol concentration rose from 3.9 ± 0.5 in the intact animals to 5.6 ± 0.9 per cent twelve days after operation. These figures are in agreement with the previous observations of Sayers and others.[8] Subsequently the cholesterol concentration declined, reaching the preoperative level in twenty-two- and twenty-eight-day animals.

Group I.—Cortisone-treated: A total of twenty-one animals from four separate experiments were used. The average adrenal weight per 100 grams

body weight (calculated on the basis of body weight immediately before the institution of treatment) was essentially the same (10.2 ± 1.4 mg.) as that of the untreated hypophysectomized controls (10.5 ± 0.8 mg.). This confirms the observation of Stebbins[4] who administered 3 mg. cortisone daily for ten- and thirty-day periods to pair-fed hypophysectomized animals. The adrenal cholesterol concentration of the cortisone-treated group (4.6 ± 1.2 per cent) was not significantly different from that of the twenty-two-day posthypophysectomy controls (3.9 ± 0.7 per cent).

TABLE I

ANIMALS	TREATMENT	BODY WT. (GRAMS)	WT. LOSS (PER CENT BODY WEIGHT)	ADRENAL WT. (PER 100 GM. BODY WEIGHT)	CHOLESTEROL (PER CENT)	
Intact (9)	None	127 ± 11		19.6 ± 2.7	3.9 ± 0.5	P = .01
12 days posthypox (5)	None	135 ± 17	0	10.2 ± 1.3	5.6 ± 0.9	P = .01
22 days posthypox (5)	None	103 ± 5	0	10.5 ± 0.8	3.9 ± 0.7	
28 days posthypox (5)	None	112 ± 12	0	12.4 ± 2.0	3.9 ± 0.7	
22 days posthypox (21)	Cortisone 5 mg. OD x 15	113 ± 18	21	10.2 ± 1.4	4.6 ± 1.2	
22 days posthypox	Hydrocortisone 5 mg. OD x 15	125 ± 14	22	9.0 ± 1.2	4.2 ± 0.8	
22 days posthypox	Corticosterone 5 mg. OD x 15	95 ± 11	24	10.1 ± 1.6	8.5 ± 2.1	P = .01
22 days posthypox (20)	ACTH 3 mg. OD x 15	104 ± 15	4	16.7 ± 3.0	6.2 ± 1.6	P = .01
22 days posthypox (15)	ACTH 3 mg. and cortisone 5 mg. OD x 15	102 ± 19	19	15.7 ± 2.9	6.4 ± 2.2	
22 days posthypox (13)	ACTH 3 mg. and hydrocortisone 5 mg. OD x 15	120 ± 15	13	13.8 ± 1.9	7.2 ± 1.2	
22 days posthypox (5)	ACTH 3 mg. and corticosterone 5 mg. OD x 15	96 ±12	19	20.6 ± 1.2	6.3 ± 1.0	P = .01

Group II.—Hydrocortisone-treated: There was no significant change in the adrenal weight from the controls and the cholesterol concentration (4.2 ± 0.8 per cent) did not differ materially from the controls (3.9 ± 0.7 per cent).

Group III.—Corticosterone-treated: The adrenal weight was essentially the same as that of the controls, but this group showed the highest cholesterol concentration of all—8.5 ± 2.0 per cent. We were surprised at these findings and are fully aware that this is at variance with what one would expect. We do not have any explanation for this finding and due to limitation of available steroid, we were unable to repeat this experiment. It is simply reported here as an observation which needs further study before any definite conclusion can be drawn.

Group IV.—ACTH-treated: A total of twenty animals in four separate groups were given ACTH in the manner described. The average adrenal weight (16.7 ± 3.0 mg. per 100 grams body weight) at the end of the treatment

was slightly less than that of the intact untreated controls (19.6 ± 2.7 mg. per 100 grams). The adrenal cholesterol concentration, twenty-four hours after the last ACTH injection, was elevated to 6.2 ± 1.6 per cent as compared to the level of 3.9 ± 0.5 per cent in untreated animals. This finding also agrees with previous observations by Sayers and associates.[8]

Group V.—Cortisone acetate plus ACTH-treated: The average adrenal weight per 100 grams body weight was 15.7 ± 2.9 mg. and is approximately the same as that of the rats treated with ACTH alone (16.7 ± 3.0 mg.).

Group VI.—Hydrocortisone plus ACTH-treated: The adrenal weight (13.8 ± 1.9 mg. per 100 grams) did not differ significantly from that of the group treated with ACTH alone, that is, 16.7 ± 3.0 mg. per 100 grams.

Group VII.—Corticosterone plus ACTH-treated: The adrenal weight of the group (20.6 ± 1.2) seems greater than that of the ACTH-treated controls as a whole, but analysis of the data indicates that it was entirely comparable to the particular group of ACTH-treated controls which were run simultaneously and which showed an average value of 19.6 ± 3.3 mg. based on five animals. Thus it may be concluded that none of these steroids inhibited the increase of the adrenal weight (i.e., regeneration) induced by ACTH treatment. There was no significant effect on the adrenal cholesterol, for the three steroid plus ACTH-treated groups yielded values of 6.4 ± 2.2 per cent, 7.2 ± 1.2 per cent and 6.3 ± 1.0 per cent, respectively, as compared to the control value of 6.2 ± 1.6 per cent.

It is interesting that each of the three steroids produced a loss of approximately 20 per cent of the total body weight in 15 days, whereas animals treated with ACTH lost only 4 per cent of their weight. When ACTH and the steroids were given conjointly, however, the weight loss did not exceed that produced by the administration of steroids alone, although these animals appeared to be in far worse physical condition at the end of the experiment than the other groups.

DISCUSSION

The adrenal atrophy found in the untreated animals killed at various times after hypophysectomy demonstrates the well-established fact that the atrophy occurs promptly and reaches a maximum in a relatively few days. There was a transitory increase of cholesterol which was evident twelve days after operation but had disappeared in twenty-one days. This interesting phenomenon, occurring in spite of the reduced weight (atrophy) of the adrenal gland, still requires elucidation.

Prolonged cortisone treatment did not further reduce the weight of the atrophic posthypophysectomy adrenal and there was no alteration of the cholesterol concentration as a result of such treatment. The two other adrenal steroids tested by us likewise failed to influence adrenal weight in hypophysectomized animals. One compound (corticosterone) did effect the cholesterol concentration.

Lewis and his colleagues[9] found that cortisone did not cause alteration in adrenal weight or adrenal cholesterol concentration in rats maintained continuously with ACTH after hypophysectomy. This is strong evidence in favor of the clinical advantages of simultaneous steroid and ACTH administration. From our point of view, however, it was important to discover whether adrenal regeneration could be brought about during cortisone therapy, and for this reason our experiments differed from those of Lewis and his co-workers, in the sense that adrenal atrophy was allowed to develop before treatment was instituted. The data we have obtained indicate clearly that adrenal regeneration induced by ACTH is unaffected by the concomitant administration of an adrenal steroid. This observation seems at first to be at variance with certain published clinical data, as those of Forsham and associates,[5] McIntosh and Holmes,[6] Wilkins and his colleagues,[7] and Engelman and co-workers,[10] who reported that the cortisone-suppressed adrenal cortex failed to show clinical response to ACTH stimulation for as long as ten days after termination of steroid therapy. Actually, these observations simply indicate that this is not an immediately reversible process.

The data obtained in the course of this study do not yield unequivocal information with respect to the basic problem of how cortical steroids produce adrenal atrophy. Lewis and his associates,[9] on the basis of the work quoted previously, came to the conclusion that this effect was entirely one of pituitary suppression by the cortisone, with consequent adrenal atrophy due to lack of endogenous ACTH. Our evidence is in agreement with such a hypothesis, but does not exclude the possibility that direct effects of steroids on adrenal tissue may also exist. Recently, Wilkins and his colleagues[7] have obtained suggestive clinical evidence of such a direct effect, but more fundamental yet are the observations recently published by Winter and his co-workers.[11] In this work, it was found that the simultaneous administration of cortisone and testosterone or cortisone and methyl androstenediol caused adrenal regeneration in the hypophysectomized rat, although none of these steroids had such an effect when employed singly. The mechanism of this synergistic direct action of steroids on the adrenal cortex is as yet unexplored.

CONCLUSIONS

1. Cortisone, hydrocortisone, and corticosterone do not reduce further the weight of the adrenal allowed to atrophy following hypophysectomy.

2. ACTH is capable of regenerating and maintaining the adrenal weight and cholesterol concentration of the adrenal of the hypophysectomized rat whether or not an adrenal steroid is given simultaneously.

3. The clinical implications of these findings, such as the possibility of avoiding the dangers attendant on the sudden discontinuation of cortisone therapy, are discussed.

4. The simultaneous administration of ACTH with the steroids will prevent adrenal atrophy.

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THE EFFECT OF (1) HYPOPHYSECTOMY ALONE AND (2) HYPOPHYSECTOMY PLUS VARIOUS COMBINATIONS OF THE STEROIDS AND ACTH UPON PHAGOCYTOSIS BY THE RETICULOENDOTHELIAL SYSTEM OF INTRAVENOUSLY INJECTED INDIA INK PARTICLES

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THE influence of the adrenal cortex on the defense mechanisms of the body has commanded increasing attention from physiologists and clinicians in recent years. Much work has been done on the immunologic and allergic mechanisms, and on the role of the lymphatic system, but, as Gordon and Katsh[1] have pointed out, the reticuloendothelial system has received scant notice. Despite the fact that a relationship between the endocrine and the reticuloendothelial systems was observed as early as 1924 if not before, adequate quantitative techniques were not developed for another twenty-five years.

In 1949, Gordon and Katsh[1] studied the histology and the phagocytic acactivity of the reticuloendothelial system by means of injected Thorotrast, a radiopaque colloidal suspension. It developed that macrophage size and activity diminished following adrenal ablation, but that they could be restored by the administration of large doses of adrenal cortical extract. Desoxycorticosterone improved the histologic appearance of the macrophages, but not their phagocytic index. Cortical extract, furthermore, hastened the transformation of fixed endothelial cells into macrophages. By contrast, little change in the reticuloendothelial system was observed following hypophysectomy. On the basis of the observed histologic changes, Gordon and Katsh felt that adrenal cortical activity exerted a direct cellular effect.

Lurie and co-workers[2] have studied this problem from another aspect. Using a quantitative technique—the weight of India ink particles retained by liver and spleen three hours after intravenous injection—they observed that rabbits treated with Cortisone (1 mg. per kilogram per day) showed decidedly greater phagocytosis than the controls. More revealing still were observations following the administration, by inhalation, of human-strain tubercle bacilli. It was found that, although phagocytosis of the tubercle bacilli was greater in the treated rabbits, these pharmacologic doses of Cortisone interfered appreciably with the capacity of the macrophages to digest the trapped bacilli.

In conjunction with other studies on the mechanism and possible prevention of the deleterious effects which sometimes accompany the therapeutic

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use of massive doses of adrenal steroids, it was decided to study the India ink uptake of the reticuloendothelial system with qualitative and quantitative techniques.

MATERIALS AND METHODS

Male white Holtzmann-Rolfsmeyer rats were used throughout. After a week on the diet recommended by Greep and Deane with water ad libitum, the animals were hypophysectomized by a technique approximating as closely as possible that of Munson and associates.* Only those animals which did not show appreciable weight change (5 grams) in the week after operation were used for subsequent experiments. All treatments were started on the seventh day after operation and continued for fifteen days. Twenty-four hours before sacrifice, the animals were imprisoned in a snug mailing tube, the tail brought out through an aperture and immersed in hot water for several minutes. This was found to make tail-vein injection of adult animals much easier. Five-tenths milliliter of a 1:10 dilution of Higgins India ink in isotonic saline was injected directly into the vein. In the ensuing twenty-four-hour period, no toxic signs were observed, although some appeared at the beginning of the second day; this was not surprising in view of the extremely elevated blood urea levels which develop soon after India ink injection.

At sacrifice, sections of liver and spleen were placed in Bouin's fluid for histologic examination, and 1 Gm. slices of tissue were subjected to quantitative India ink assay. The method we have developed is based on the standard liver glycogen technique; the tissue is placed in a 15 ml. centrifuge tube, 5 ml. of 30 per cent KOH is added, and the material digested completely on the steam bath. The tube is cooled, centrifuged, and the supernatant drained off. Five milliliters of KOH are added to the precipitate which is stirred up; the tube is heated briefly, cooled, and centrifuged once more. After thorough draining, about 10 ml. of N sulfuric acid is added to the tube and the material digested on the steam bath for an hour. The tube is cooled, the volume adjusted to 10.0 ml., and one drop of any commercial sulfonated-oil detergent (Joy) added. The material is transferred to a Ten Broeck grinder and the agglomerates of carbon particles are thus easily brought into a fine dispersion. The centrifuge tube is rinsed out once with this suspension to make certain that no particle aggregates are left behind. The density of the suspension is determined quantitatively in a colorimeter, using yellow light (590 mμ) to eliminate interference by traces of yellow pigments which may remain. Clear water is used as the 100 per cent transmission standard. Serial dilutions of India ink suspensions yield a linear curve on the Leitz-Rouy colorimeter.

The present data do not include quantitative measurements on spleen India ink uptake as these organs, which are greatly diminished in size by the treatment, did not provide enough for histologic as well as for quantitative study.

RESULTS

Group I. The Effect of Hypophysectomy on the India Ink Uptake as Compared to Unoperated Rats (Table I).—It was surprising to observe that the India ink uptake increased 30 to 40 per cent following hypophysectomy and that this effect persisted up to twenty-eight days after operation. These results are all statistically significant, $p = 0.01$ or less in all instances. We have no explanation for this phenomenon, which was contrary to expectation.

Group II. The Effect of Various Steroids—Cortisone, Hydrocortisone, and Corticosterone—on India Ink Uptake (Table II).—The appropriate control group for these animals is that which was sacrificed twenty-two days after opera-

*We are deeply grateful to Dr. Paul Munson of Harvard Dental School, and his coworkers, for his help in demonstrating his technique of hypophysectomy.

tion (seven days' rest, fifteen days' treatment). The administration of massive doses of these steroids returned the India ink uptake to the level but not below that of the intact normal animal.

Group III. The Effect of ACTH Alone and When Given Simultaneously With Cortisone, Hydrocortisone, and Corticosterone (Table III).—ACTH alone restored the adrenals to their prehypophysectomized weight and also returned

TABLE I. THE EFFECT OF HYPOPHYSECTOMY ON LIVER INDIA INK UPTAKE

ANIMALS	TREATMENT	ADRENAL WT. (MG. PER 100 GM. BODY WEIGHT)	LIVER INDIA INK UPTAKE (ARBITRARY UNITS)	
Intact	None	19.6 ± 2.7	110 ± 17 (12)	P = .01
12 days posthypox (5)	None	10.2 ± 1.3	153 ± 27 (4)	
22 days posthypox (5)	None	10.5 ± 0.8	146 ± 25 (5)	
28 days posthypox (5)	None	12.4 ± 2.0	141 ± 24 (5)	

Posthypox = after hypophysectomy.

TABLE II. THE EFFECT OF CORTISONE, HYDROCORTISONE, AND CORTICOSTERONE ON LIVER INDIA INK UPTAKE

ANIMALS	TREATMENT	ADRENAL WT. (MG. PER 100 GM. BODY WEIGHT)	LIVER INDIA INK UPTAKE (ARBITRARY UNITS)	
Intact	None	19.6 ± 2.7	110 ± 17 (12)	
22 days posthypox (5)	None	10.5 ± 0.8	146 ± 25 (5)	P = .01
22 days posthypox (21)	Cortisone 5 mg. OD x 15	10.2 ± 1.4	104 ± 26 (16)	
22 days posthypox	Hydrocortisone 5 mg. OD x 15	9.0 ± 1.2	115 ± 25 (6)	
22 days posthypox	Corticosterone 5 mg. OD x 15	10.0 ± 1.6	105 ± 21 (5)	

TABLE III. THE EFFECT OF ACTH ALONE AND COMBINED WITH VARIOUS STEROIDS UPON LIVER INDIA INK UPTAKE

ANIMALS	TREATMENT	ADRENAL WT. (MG. PER 100 GM. BODY WEIGHT)	LIVER INDIA INK UPTAKE (ARBITRARY UNITS)
Intact	None	19.6 ± 2.7	110 ± 17 (12)
22 days posthypox (5)	None	10.5 ± 0.8	146 ± 25 (5)
22 days posthypox (20)	ACTH 3 mg. OD x 15	16.7 ± 3.0	107 ± 16 (18)
22 days posthypox (15)	ACTH 3 mg. and Cortisone 5 mg. OD x 15	15.7 ± 2.9	107 ± 20 (11)
22 days posthypox (13)	ACTH 3 mg. and hydrocortisone 5 mg. OD x 15	13.8 ± 1.9	100 ± 25 (15)
22 days posthypox (5)	ACTH 3 mg. and corticosterone 5 mg. OD x 15	20.6 ± 1.2	107 ± 8 (3)

the India ink uptake to that of the unoperated animals. Simultaneous administration of ACTH and steroids which returned the adrenals to normal size and subjected the animals to enormous overdosage, depressed the India ink uptake from the level seen in the controls to that observed in intact animals, but not below this level.

DISCUSSION

Our own observations that large doses of various steroids did not depress the India ink below that of the intact animal, and those of Lurie and associates[2] that animals treated with cortisone showed a decidedly greater phagocytosis for India ink and tubercle bacilli than the normals, are suggestive evidence that cortisone and similar compounds do not depress the phagocytic ability of the macrophages. The India ink uptake was approximately the same whether the animals received ACTH alone, or simultaneously with the various steroids, or received the steroids alone, and was approximately the same as that of the intact animals in all instances. Gordon and Katsh, using Thorotrast, reported that the "administration of whole adrenal cortical extract results in the appearance of greater numbers of macrophages containing increased amounts of colloidal agent in the majority of the reticuloendothelial organs of both the adrenalectomized and hypophysectomized animal."[1] These observations, however, may apply only to Thorotrast and India ink particles and should not be generalized. The reticuloendothelial system is known to behave differently with different substances, and our results with India ink do not necessarily agree with those obtained by using Thorotrast or other materials. Moreover, no one substance can or should be interpreted as representing the totality of reticuloendothelial phagocytosis as it occurs in vivo.

If cortisone lowers the resistance to certain infections and, as pointed out previously, if it does not reduce the phagocytic ability of the macrophages, then there must be another reason for the lowered resistance. Perhaps this reason may be found in Lurie and colleagues' observation[2] that following the inhalation of human tubercle bacilli, the phagocytosis of the bacilli was increased but the ability of the macrophage to digest the bacilli was appreciably diminished in the cortisone-treated animal. This phenomenon may more truly represent the physiologic pattern. On the basis of their observations, it appears that the resistance mechanism does not depend on phagocytosis alone and that the phagocytic activity of the macrophage may be as great or greater in the cortisone-treated animal as in the intact animal. Therefore, the fact that the India ink uptake was approximately the same in hypophysectomized animals receiving large doses of ACTH or the steroids alone or simultaneously does not necessarily mean that the resistance of the three groups is the same. Since the administration of ACTH to the hypophysectomized animal will return the adrenal weight to approximately that of the intact animal, it raises the question as to whether ACTH administered simultaneously with the steroids will maintain the ability of the macrophage to digest the invading

organism on the same level as that of the intact animals. If this is a fact, then perhaps the simultaneous administration of ACTH with the steroids will maintain a normal resistance in chronic-cortisone-treated patients.*

CONCLUSIONS

Uptake of intravenously injected India ink particles was used to study the effect of steroids on phagocytosis by the reticuloendothelial system.

The India ink uptake was increased in the hypophysectomized animal.

The administration of large doses of cortisone, hydrocortisone, and corticosterone reduced the level of the uptake in the hypophysectomized animals but not below that of the intact, untreated animals.

The simultaneous administration of ACTH and cortisone, hydrocortisone or corticosterone did not alter the effects seen with the administration of the steroids alone.

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*This is the subject of another experiment now being carried out.

ZONE ELECTROPHORESIS IN THE STUDY OF SERUM LIPOPROTEINS

I. Methods and Preliminary Results

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IN RECENT years much attention has been directed toward the serum lipids and lipoproteins and their role in the etiology of atherosclerosis. A number of different techniques have been employed in the study of this problem. Gofman and colleagues[1] have pioneered in the use of the ultracentrifuge to estimate serum lipoprotein fractions by their sedimentation velocity. Turner and associates[2] have used the quality ultracentrifuge and mechanically separated the centrifugate into a number of fractions for chemical analysis. Barr and associates[3, 4] have determined the lipids in the serum fractions separated by the alcohol precipitation methods developed earlier by Cohn.[5] Others have used the free electrophoresis of the serum proteins before and after the extraction of the lipids[6] or have used the serum lipid levels and cholesterol-phospholipid ratio as an index of the atherosclerotic state.[7-9]

Since its introduction for protein analysis by Cremer and Tiselius[10] and Wieland,[11] zone electrophoresis on filter paper has been used to study serum lipid and lipoprotein fractions. Raynaud and associates[12] and Malmros[13] have shown with this method that changes in the total lipid pattern occur in cardiovascular disease. Investigations have been proceeding in this laboratory for some time on the serum protein distribution in diseased states using the free electrophoresis. We thought that further information could be gained by using, in addition, the zone electrophoresis for the determination of the lipoprotein distribution. Our earlier results with filter paper electrophoresis were not entirely satisfactory even for the determination of total lipid distribution. In order to determine adequately the partition of cholesterol and phospholipid, several sheets of filter paper have to be used simultaneously and we experienced difficulty in securing an even migration in all the sheets. We obtained much more satisfactory results using a starch-supported medium as described by Kunkel and Slater.[14] With slight modifications of their technique, as described later, we were able to obtain satisfactory curves showing the distribution of cholesterol and organic phosphorus between the protein fractions. The method should also be applicable to other procedures when it is desired to use larger quantities than can be handled by filter paper methods.

APPARATUS AND METHOD

A sketch of the apparatus is shown in Fig. 1, *A*. In this figure, *1* is the plastic holder for the starch, shown in cross section in Fig. 1, *B*. The plastic parts are all made of Lucite or Plexiglas acrylic plastic. The starch holder consists of two strips ¼ inch thick and meas-

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uring 3½ by 18 inches separated by two ⅜ inch square rods. Three ⅛ inch holes are drilled along each side and plastic pins inserted to align the parts as shown. The ends of the holder are open for electrical contact.

Parts *2* are 2½ by 4 by 6 inch commercial cellulose sponges with a portion cut out about 1 inch deep into which the holder, *1*, may be inserted, and cut to fit snugly in the containers, *3*. These are cylindrical plastic containers about 5 inches in inside diameter and 5 inches high with a portion cut from each wall to allow the holder, *1*, to project inward as shown. They are made from stock cylindrical plastic with bottoms cemented on. These have tops which may be placed on to reduce evaporation. The containers are connected by small outlets near the bottom and a length of ¼ inch rubber tubing for the purpose of equalizing the liquid levels. It is not necessary to close this tubing during a run. Parts *4* are electrolyte bridges of 1 inch glass tubing with a small side tube at the top which is closed by a small section of rubber tubing and a screw clamp and used for ease in filling the syphons. Parts *5* are other plastic containers similar to *2*; glass jars would serve equally well here.

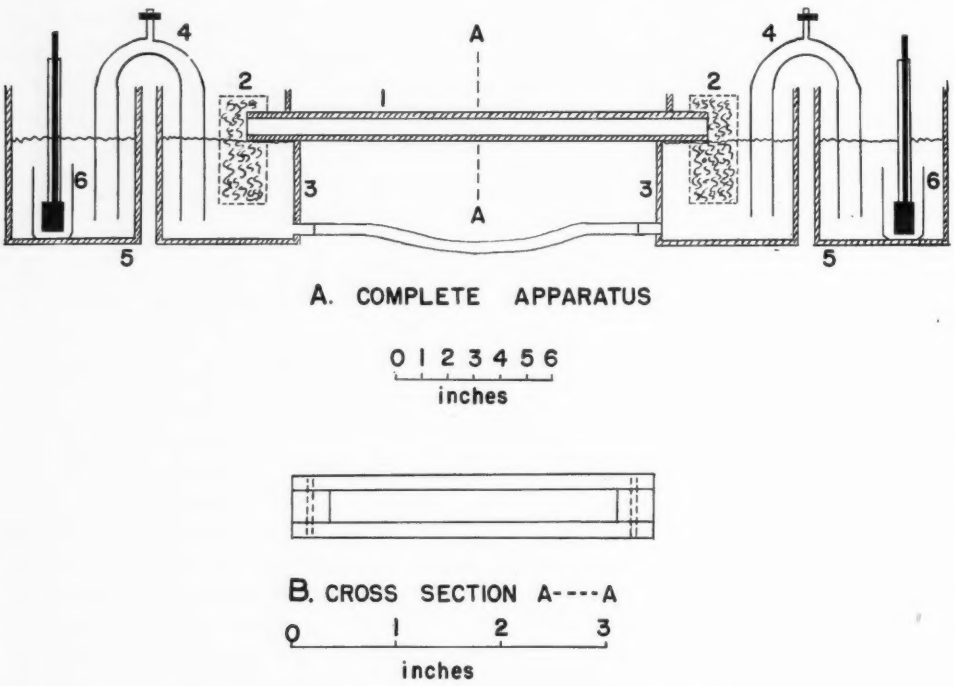


Fig. 1.—Zone electrophoresis apparatus (schematic diagram).

Parts *6* are silver electrodes placed in 50 ml. beakers resting on the bottom of the container. The electrodes are similar to those furnished with the Aminco Electrophoresis Apparatus.* They consisted of a pure silver sheet 1 by 6 inches and 0.005 inch thick, attached to a hollow silver tube and rolled into a coil. The silver tube is protected by an outer glass tube except at the top where electrical contact is made. Before using for the first time the electrodes are coated with silver chloride and passing a current of approximately 50 ma. for twenty-four hours from a graphite or platinum anode. If the electrodes are kept in potassium chloride solution when not in use and reversed after each experiment, that is, a given electrode is made the cathode in one experiment and the anode in the next run, they will retain their plating for several months.

*American Instrument Company, Catalogue No. 5-8770.

To assemble the apparatus, a piece of waxed paper is placed over the bottom plate of the starch holder (the waxed paper facilitates the final removal of the starch) and the square side pieces are added and held in place with the plastic pins. The potato starch was made into a thick paste with Veronal buffer of 0.1 ionic strength (3.69 Gm. of barbituric acid and 20.62 Gm. of sodium barbital, made up to two liters and adjusted to a pH of 8.6). We have used Amend potato starch* as suggested by Kunkel[15] as being the most satisfactory for zone electrophoresis, but have found some variation between different lots of the material. The purified potato starch obtained from the Fisher Scientific Co. was more uniform but also more expensive. The starch paste is placed evenly in the holder, the ends of which are temporarily closed by butting against heavy pieces of blotting paper. After the excess moisture has been removed by blotting paper, any excess starch projecting above the sides is removed. Then a cut is made with a knife across the starch block 5 inches from one end. The cut does not extend entirely across the starch block, about ¼ inch at each side being undisturbed. The cut is enlarged somewhat at the top to a narrow V without removing any starch. Two and one-half milliliters of serum is now distributed evenly along the cut from a pipette. After all the serum has been absorbed, the V is carefully pressed together with a spatula. A second piece of wax paper is placed on top of the starch and the second plate placed in position. The plates may be held together with several small C-clamps or with strips of adhesive tape applied completely around at several points. The containers are then filled with buffer and the starch holder placed between the sponges.

The whole apparatus is made to fit in the bottom of a large refrigerator so that the electrophoresis is carried out at a low temperature. The bridges are filled and the electrodes placed in position. With the aid of a syringe and hypodermic needle 2N potassium chloride solution is slowly introduced through the hollow electrode tube to just completely cover the electrodes in the beakers. The use of the hollow electrode tubes may be an unnecessary refinement as the potassium chloride solution could be successfully placed in the beakers merely by the use of a syringe and a sufficiently long needle. A current of 30 ma. is then passed through the solution at a potential of some 700 to 800 volts for from sixteen to eighteen hours. An unregulated power supply may be readily constructed but we have found it convenient to use a regulated power which keeps the current constant in spite of changes in resistance which occur in the solution. In this way the electrophoresis may be safely carried on overnight without any supervision once the proper conditions have been established. A wiring diagram for such a power supply may be found in the appendix to this article.

The extent of migration can usually be followed by observing the major yellow protein band as it travels along the starch. When this band has traveled 8 or 9 inches, the electrophoresis is complete. The starch holder is then removed from the apparatus and the top and sides are taken off. The starch block is then carefully cut into thirty-six equal ½ inch sections which are transferred quantitatively to consecutively numbered 30 ml. beakers. To each beaker is then added 3 ml. of 1 per cent saline and the starch mixed well with a small stirring rod which is left in the beaker. After settling, 0.5 ml. of the supernatant is pipetted off for the determination of protein.

The protein is determined with Folin's reagent.[16] To the 0.5 ml. aliquot in a test tube graduated at 10 ml. is added 3 ml. of 1.2 M sodium carbonate and 1 ml. of the reagent. The solution is then diluted to 10 ml., mixed well, and allowed to stand for fifteen minutes. After filtering through a small filter to remove traces of starch, the solution is read in a colorimeter.

The major portion of the starch remaining in the beaker is then dried in a vacuum oven at 40° C. In later work, particularly with repeated determinations on the same subject, we dispensed with the protein determination but found that the mixing of the starch blocks with 1 ml. of saline before drying produced a better dried product. The starch is usually removed from the oven twice during the drying process and broken up with the small stirring rods remaining in the beakers. The dried starch is then extracted with a

*Obtained from the Amend Drug and Chemical Co., New York City.

3:1 mixture of ethyl alcohol and ether. About 20 ml. of warm alcohol-ether is mixed well with the starch in the beaker, covered with a watch glass and allowed to stand in a warm place for several hours. The starch is then filtered off on a sintered glass filter using moderate suction, collecting the filtrate in a tube graduated at 20 ml. The starch is then washed with several successive small portions of warm alcohol-ether until the final volume of filtrate is 20 ml. Five milliliters of the filtrate is taken for phosphorus analysis, the remainder for cholesterol analysis.

For the phosphorus analysis the 5 ml. aliquots are pipetted to test tubes graduated at 5 and 10 ml. The alcohol-ether is carefully evaporated, at first by immersing the tubes in warm water and finally in an oven. The organic material is then destroyed by placing the tubes in a sand bath at 150° C. and adding 6 drops of 70 per cent perchloric acid. The digestion is continued until the acid mixture is clear and colorless. The tubes are then cooled, 1 ml. of water added, and the phosphorus determined by the A. O. A. C. colorimetric method.[17] The method is followed in detail except that the final volume is 10 ml. instead of 100 ml., the tubes are diluted to 7.5 ml. before adding the reagent (equidistant between the 5 and 10 ml. marks), and the quantities of the reagents are reduced proportionately. In running a large number of samples we found it very convenient to add the 1 ml. of molybdenum reagent with an electrically operated automatic pipetting device. Blanks on the reagents and a set of standards were run with every set of analyses. Great care must be taken to eliminate traces of phorphorus from other sources. We found it difficult to remove the last traces of phosphate when the glassware was washed with our usual laboratory detergents which contained large amounts of phosphates. We finally adopted a policy of cleaning the test tubes only with sulfuric acid-chromate solution followed by thorough rinsing with distilled water.

The cholesterol was determined in the remaining 15 ml. of extract. This was evaporated to dryness and extracted several times with successive small portions of hot chloroform to a total final volume of chloroform extract of 5 ml. when cold. To this was added 5 ml. of a chilled mixture of acetic anhydride and concentrated sulfuric acid (20:1). The tubes were read in the Beckman exactly eighteen minutes after the addition of the reagent at 25° C. Blanks and a set of standards were also included in each set of analyses.

Total cholesterol and phospholipid of the serum were also determined by adding 1 ml. of serum to about 40 ml. of warm alcohol ether. The mixture was allowed to stand for several hours with occasional shaking, cooled, and diluted to 50 ml. After mixing, the solution is filtered and aliquots taken for cholesterol (10 ml.) and phosphorus (5 ml.) by the methods outlined previously. Phospholipid was calculated by the use of the factor $P \times 25 =$ phospholipid. For plotting the curves the total amount of cholesterol and phospholipid extracted from each segment was calculated. In the curves shown here the initial position of the serum was usually at segment No. 26 and the migration was to the left with the final alpha peak in the neighborhood of segment No. 6.

RESULTS

Fig. 2 gives the results obtained on the serum from a healthy young adult man. The protein curve is shown in Fig. 2, *A*. Since only the relative amounts of the various protein fractions are of interest, the optical density figures are plotted directly. The cholesterol and phospholipid curves are given in Fig. 2, *B*. Here the results are plotted as micrograms of cholesterol or phospholipid in the total extract from each segment. It will be noted that in the normal individual the cholesterol and phospholipid associated with the lipoprotein are divided into two main fractions. Under our experimental conditions one fraction migrates at approximately the same rate as the albumin and may be identified with the alpha lipoprotein fraction. The other main fraction migrates with the beta-2 globulin and contains the beta lipopro-

teins. There is also an intermediate fraction which is low in normal individuals but which we have found to be markedly elevated in some pathologic states. We have used the protein curves obtained in the zone electrophoresis only to check the position of the lipid fraction. For accurate analysis of the changes in protein distribution we use the free electrophoresis.[18] Since the position of the lipid fractions with respect to the protein fractions is nearly the same in all cases, the protein curves are not shown in the other figures.

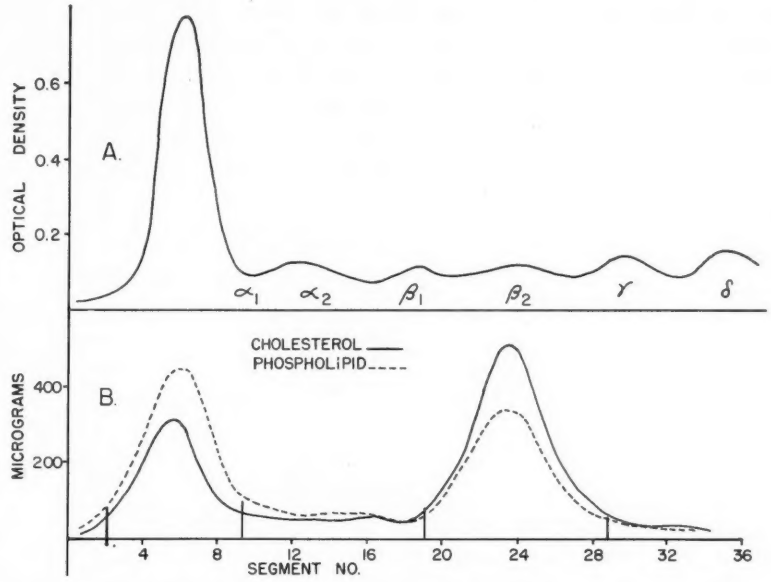


Fig. 2.—*A*, Protein distribution—normal subject. *B*, Cholesterol and phospholipid distribution—normal subject.

If we calculate the relative amounts of cholesterol and phospholipid in the various fractions as marked off in Fig. 2, *B*, we find that in the normal individual we have, on the average, approximately 29 per cent of the cholesterol in the alpha fraction, 9 per cent in the intermediate fraction, 54 per cent in the beta fraction, and the remaining 8 per cent in the two "tails". According to Barr,[19] in normal human plasma approximately 30 per cent of the cholesterol is in the alpha fraction and 70 per cent in the beta lipoproteins. An exact comparison with our results is not possible because of the difference in methods, but a similarity will be noted if we combine our intermediate and beta fractions for the comparison.

In comparing the results from different subjects the general shape of the curve is of great interest. In comparison of numerical data we have used the relative percentages of cholesterol and phospholipid in the three main fractions, neglecting the "tails" since we are here interested only in the relative amounts. We have also computed the absolute amounts of cholesterol and phospholipid in each fraction from the percentages and the total serum choles-

terol. In this the amount in the "tails" is not neglected. These latter data are of less accuracy since they are obtained by multiplying the total cholesterol (or phospholipid) by the percentage in the fraction, each of which is subject to experimental error. They are still of value in comparing the values from different subjects all obtained by the same technique.

Figs. 3 to 7 show typical curves obtained from subjects with different pathologic conditions. Table I gives the corresponding numerical data on the same subjects. In the table are given the relative percentages of cholesterol and phospholipid in the three fractions, viz., alpha, intermediate, and beta, as well as the total serum cholesterol and phospholipid and the computed absolute amounts in each fraction. No. 2 in the table gives the numerical data on the normal subject shown in Fig. 2. In this subject the alpha fraction contains 32 per cent of the cholesterol or 51 mg. per cent (i.e., 51 mg. of cholesterol in the alpha fraction from 100 ml. of serum) and 42 per cent of the phospholipid or 80 mg. per cent.

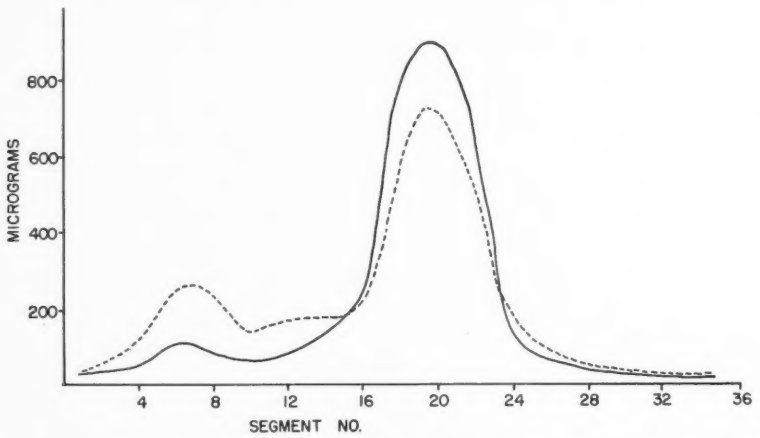


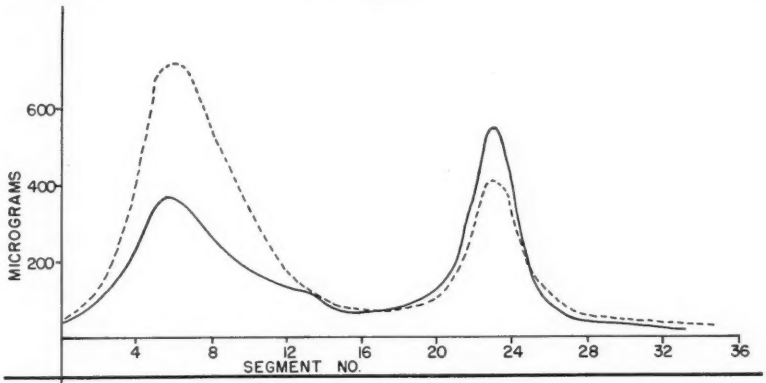
Fig. 3.—Lipoprotein curves—69-year-old male with hypertensive cardiovascular disease. Solid line indicates cholesterol; broken line, phospholipid.

Fig. 3 and No. 3 in the table are the data obtained on a 69-year-old man with hypertensive cardiovascular disease. He had a cerebral thrombosis two years previously and has since shown a progressive deterioration in clinical status. Not only is the total serum cholesterol and phospholipid elevated, but percentage in the alpha fraction is markedly decreased so that the absolute amount in the alpha fraction is lower than normal in spite of the elevation of serum levels. Correspondingly the amount of lipids in the beta fraction is greatly increased as is usually found in severe atherosclerosis or cardiovascular disease.

Fig. 4 and No. 4 in the table refer to a 56-year-old woman with hyperthyroidism. In this instance the increase in lipids is found in the alpha fraction. The total cholesterol and phospholipid are elevated but the amount in the beta fraction is nearly the same as in Subject 2.

Fig. 5 and No. 5 in the table are the results obtained from a 73-year-old woman. She has minimal evidences of arteriosclerosis but has had diabetes for a number of years. We note some increase in the total cholesterol and phospholipid, and a decrease in the percentage of lipids in the alpha fraction. It will be noted that the absolute amount of cholesterol and phospholipid in the alpha fraction is about the same as in the normal subject, No. 2. The increase in lipids is then entirely in the beta fraction.

Fig. 4.



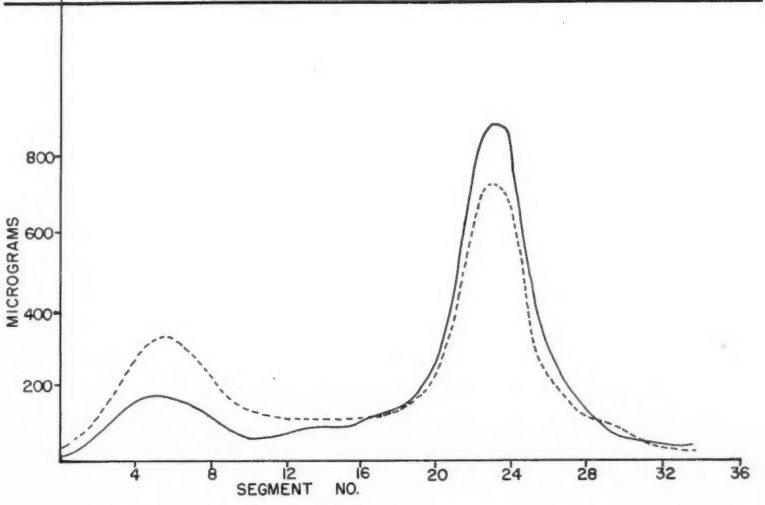


Fig. 5.

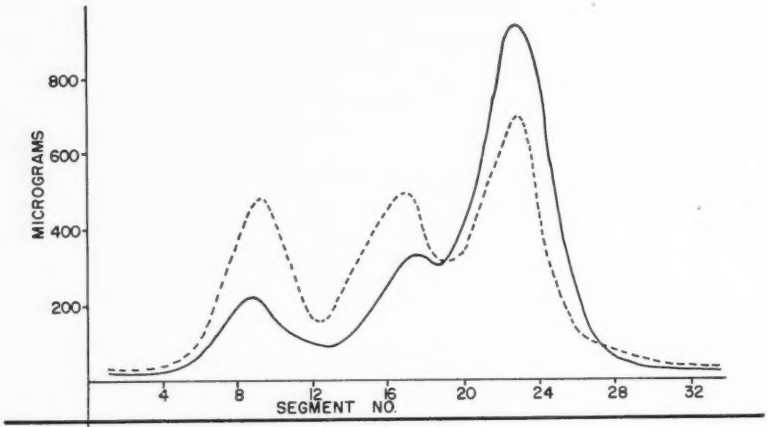
Fig. 4.—Lipoprotein curves—hyperthyroidism. Solid line indicates cholesterol; broken line, phospholipid.

Fig. 5.—Lipoprotein curves—73-year-old female in good health. Solid line indicates cholesterol; broken line, phospholipid.

Figs. 6 and 7 and the corresponding numbers in the table are the two results on the same patient taken approximately two months apart. This patient had a history of myocardial infarction with the electrocardiogram showing evidences of myocardial damage. He was known to be sensitive to an increased fat intake. Fig. 6 is the curve obtained when the patient was

on an uncontrolled diet. Fig. 7 shows the results obtained two months later after rest, and treatment with low-fat diet, iodide, and estrogens. Note that the second curve is much closer to normal. In Fig. 6 there is an unusually large percentage of lipids in the intermediate fraction. The exact significance of this type of curve has not yet been established. We have not yet observed it in a sufficient number of cases to establish a definite correlation with any

Fig. 6.



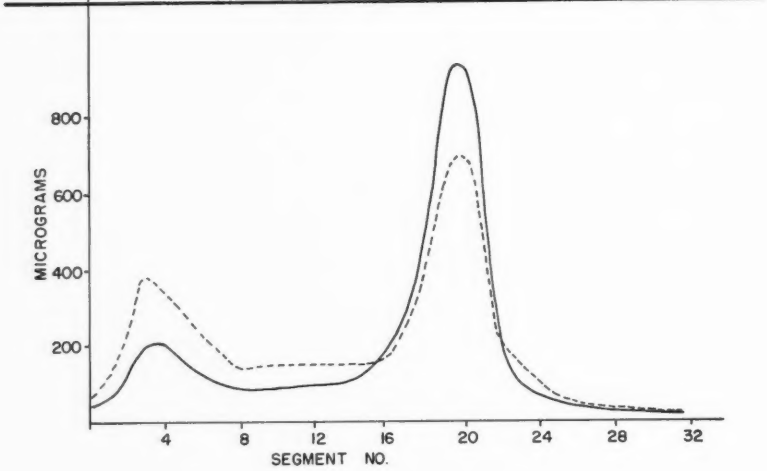


Fig. 7.

Fig. 6.—Lipoprotein curves—53-year-old male subject after myocardial infarction. Solid line indicates cholesterol; broken line, phospholipid.

Fig. 7.—Lipoprotein curves—subject of Fig. 6 after treatment. Solid line indicates cholesterol; broken line, phospholipid.

clinical state, except that all the patients in which it has been observed have definite evidences of progressive cardiovascular disease. In this patient after treatment there was a decrease in the total serum cholesterol and phospholipid, but, as is shown in the table, most of this was due to a decrease in the amount in the intermediate fractions; the amount in the other fractions remained relatively constant.

TABLE I. CHOLESTEROL AND PHOSPHOLIPID DISTRIBUTION

SUBJECT	PER CENT IN FRACTIONS			AMOUNT IN FRACTIONS (MG.)			TOTAL IN SERUM (MG.)
	ALPHA	INTER-MEDIATE	BETA	ALPHA	INTER-MEDIATE	BETA	
			Cholesterol				
No. 2	32	10	58	51	16	94	173
No. 3	7	10	83	23	31	274	345
No. 4	48	15	37	119	37	91	260
No. 5	16	15	69	49	45	213	336
No. 6	14	21	65	49	72	226	360
No. 7	18	10	72	51	27	199	290
			Phospholipid				
No. 2	42	11	37	90	22	78	212
No. 3	15	16	69	51	53	240	373
No. 4	65	11	24	213	37	80	352
No. 5	28	19	53	90	59	170	353
No. 6	28	30	42	111	117	164	415
No. 7	32	15	53	101	45	164	335

	ALPHA	INTER-MEDIATE	BETA	TOTAL
	Cholesterol/Phospholipid Ratio			
No. 2	.57	.72	1.20	.82
No. 3	.45	.59	1.14	.92
No. 4	.56	1.00	1.14	.74
No. 5	.54	.76	1.25	.95
No. 6	.44	.62	1.38	.67
No. 7	.50	.60	1.21	.87

In studying free electrophoresis protein patterns, Bernfeld[20] states that the general and detailed morphologic appearance of the curves often yields valuable information which cannot be obtained by calculating the relative amounts of the proteins, and that the pattern from any individual has its own specific characteristics. Our lipoprotein zone electrophoresis curves are somewhat less complex than the free electrophoretic curves, yet the applicability of Bernfeld's statement to them is demonstrated in Fig. 8. The cholesterol curves alone are drawn as they are sufficient for demonstration without making the figure too complicated as it would be if the phospholipid curves were also added.

In Fig. 8, curve *1* is from a normal young adult and is similar to the cholesterol curve in Fig. 1, *B*. All the other curves in the figure are from elderly subjects between the ages of 70 and 80 years. Curve *2* is from a female subject who had received estrogens for several years in connection with other studies, and curve *3* is from a male subject who had been receiving thyroid and estrogen for approximately two months. In both the percentages of cholesterol in the three main fractions were 16, 11, and 73 per cent respectively. Although these percentages are the same for the two subjects, distinct differences in the shape of the curves are noted.

In curves *4*, *5*, and *6* from three different individuals, the percentages of cholesterol in the three main fractions were 16, 14 and 70 per cent, respectively, for all three subjects. Curve *4* was from a diabetic woman in whom the disease was fairly well controlled with insulin and the total cholesterol was 335 mg. per cent. Curve *5* was from a male subject who had received thyroid and estrogen for approximately three months. His total serum cholesterol

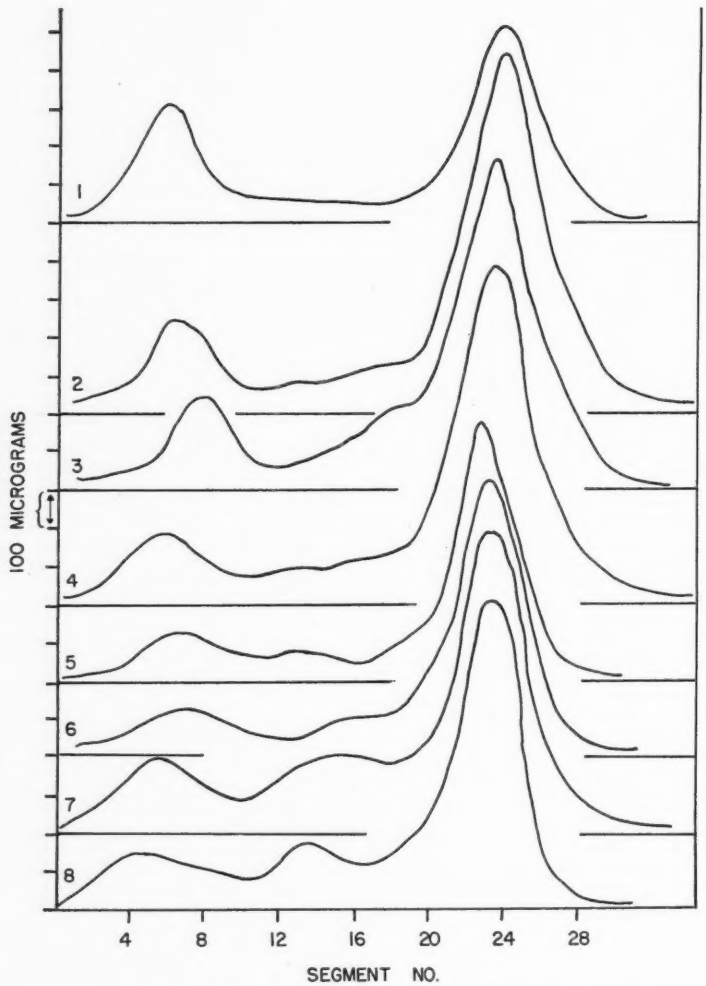


Fig. 8.—Comparison of cholesterol distribution curves (for explanation see text).

was 250 mg. per cent. This subject suffered a cerebral hemorrhage some forty days after the above curve had been made. Curve *6* was from a male subject in good health. His total cholesterol was 210 mg. per cent. The differences in the shape of the curves is readily seen.

Curves *7* and *8* are from the same male subject. His total cholesterol was 285 mg. per cent and the percentages of cholesterol in the three fractions were 18, 18, and 64 per cent, respectively. The total cholesterol and the distribution between the fractions did not change significantly in the interval between the time the two curves were made, which was about three months. Although the migration has proceeded somewhat further in one curve than in the other, the general similarity of two curves is readily apparent.

The cholesterol-phospholipid ratio in the serum has been used by others as an index of the atherosclerotic state, as mentioned previously. The relation of this ratio to the zone electrophoresis data can be seen from Table I. Numbers 3, 5, and 7 have a cholesterol-phospholipid ratio higher than the normal (No. 2) because the percentage of lipid in the beta fraction (which contains a greater proportion of phospholipid) is greater than normal and in No. 4 the cholesterol-phospholipid ratio is low because of the large amount of lipid in the alpha fraction. The changes in the cholesterol-phospholipid ratio then reflect changes in the distribution of the lipids between the different electrophoretic fractions. If there are more than two fractions the distribution of lipids between the fractions could conceivably change without changing the total cholesterol-phospholipid ratio, hence the former data would be much more valuable. This is also illustrated by curve No. 6 in which the ratio is lower than normal but the distribution of lipids is marked abnormal though higher ratios are usually considered the mark of abnormality.

Antonini and associates[21] have recently published an interesting review of their work on the use of paper electrophoresis in the study of atherosclerosis. Although they could distinguish several different lipoprotein fractions, for most purposes they used the ratio of the total lipid in the beta fractions to that in the alpha fractions. They found this to be a more valuable index of the atherosclerotic state than the cholesterol-phospholipid ratio or the total cholesterol. With the electrophoresis on the starch medium one determines the distribution not only of the total lipid, but also of the cholesterol and lipid phosphorus separately between the different fractions. Another possibility which is available with the starch electrophoresis and which is now being investigated in this laboratory, is the distribution of the free and esterified cholesterol between the different lipoprotein fractions, as it has been suggested[7, 22, 23] that the two forms play different roles in the transportation and deposition of cholesterol. It can be seen that the zone electrophoresis on a starch medium should prove a very valuable tool in the investigation of the blood lipids.

APPENDIX

The following are the components for the power supply shown in Fig. 9. For convenience the catalogue number of several of the components are given though equivalent models by other manufacturers could be used.

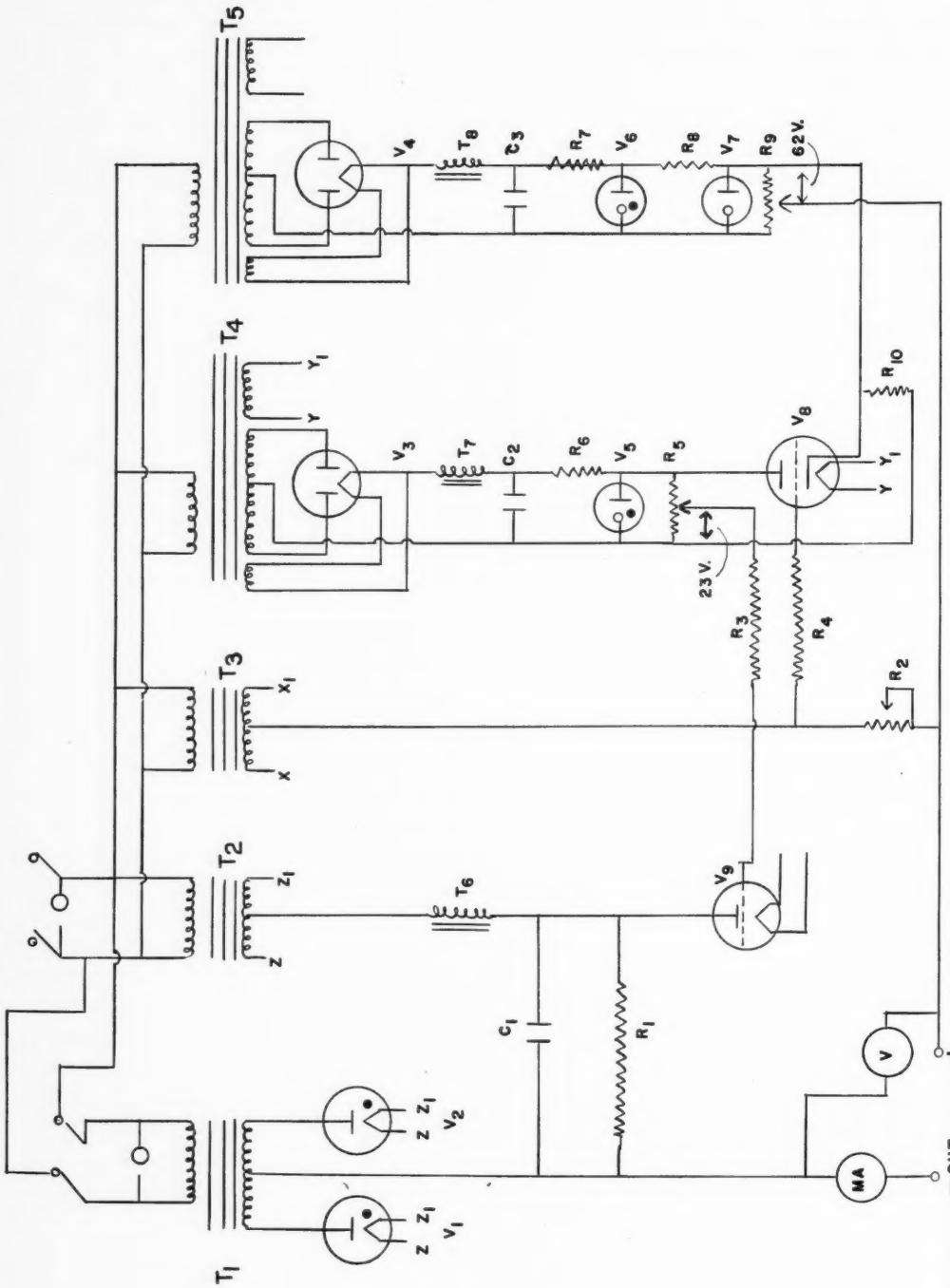


Fig. 9.—Wiring diagram.

T-1	Plate Transformer, 1075-0-1075 V., 110ma.	Thordarson No. T-21P93
T-2	Filament Transformer, 2.5 V., 10 A.	Thoradarson No. T-21F02
T-3	Filament Transformer, 10V., 5 A.	Thordarson No. T-21F-18
T-4, T-5	Power Transformers, 275-0-275 V., 50 ma.	Thordarson No. T-22R01
T-6, T-7, T-8	Filter Chokes, 10 hy., 110 ma.	Thordarson No. C10001
C-1	Condenser, 4 microfarad, 2000 V.	
C-3, C-4	Electrolytic Condenser, 16 microfarad, 400 V.	
V-1, V-2	Type 816 Vacuum Tube	
V-3, V-4	Type 5Y3GT Vacuum Tube	
V-5, V-6	Type 0D3/VR150 Voltage Regulator Tube	
V-7	Type 0A3/VR75 Voltage Regulator Tube	
V-8	Type 6F5 Vacuum Tube	
V-9	Type 8005 Vacuum Tube	
MA -	Milliammeter, 0-50 ma.	
V	Voltmeter, 0-1000 Volts	
R-1	Resistor, 360,000 Ohm, 20 watts (six 60,000 Ohm, 5 watt resistors)	
R-2	Rheostat, 10,000 Ohms, 25 watts	
R-3	Resistor, 1 megohm, 1 watt	
R-4	Resistor, 500,000 Ohm, 1 watt	
R-5	Tapped Resistor, 12,000 Ohms, 10 watts, tapped at 23 volts as shown	
R-6	Resistor, 1,500 Ohms, 10 watts	
R-7	Resistor, 2,000 Ohms, 10 watts	
R-8	Resistor, 4,00 Ohms, 10 watts	
R-9	Tapped Resistor, 10,000 Ohms, tapped 62 volts from positive side as shown	
R-10	Resistor, 750,000 Ohms, 1 watt	

Fig. 9.—Wiring diagram.

This power supply was adapted from that used in the American Instrument Company's "Portable" electrophoresis apparatus.

Since the tubes should be allowed to warm up before the high voltage supply is applied, an additional switch and pilot light is supplied for the high voltage transformer as indicated. The output current is regulated by means of the rheostat, R-2.

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INCREASE IN HYPERSENSITIVITY LESIONS OF PARABIOSIS INTOXICATION AFTER ADRENALECTOMY

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THE observation of unanticipated phenomena is one of the charms of biologic experimentation. The purpose of this work is to report and attempt to explain a striking increase in certain cardiovascular, glomerular, and hematopoietic lesions found in hemiadrenalectomized parabiotic rats, compared to parabiotic pairs with the normal complement of adrenal glands.

The experiments originally were designed to test whether irradiation of one parabiotic rat would mobilize substances producing pathologic changes in its protected partner.[1] Results were negative. To examine the role of adrenal function in radiation sickness, 72 pairs had both adrenals removed from one parabiont some time before x-ray exposure. Evidences of disturbed adrenal function became visible histologically in this group only when the one pair of adrenals serving both partners had been irradiated with relatively high doses.[1] Both in normal and hemiadrenalectomized parabionts after irradiation there were also indications of various other endocrine changes, which were more numerous and striking in hemiadrenalectomized pairs.[2]

In approximately 30 per cent of the total series of 253 rat pairs, signs of biologic incompatibility developed. Such events have been known to occur since the early days of experimental parabiosis, and are generally termed parabiosis intoxication.[3] Pathologically, abnormalities observed included failure of healing of the skin juncture, valvular endocarditis, proliferative glomerulonephritis, occasionally arteritis, and the reticuloendothelial and hematopoietic alterations found in acquired hemolytic anemia. In the 72 hemiadrenalectomized parabiont pairs, lesions of parabiosis intoxication were found in 82 per cent, over 2.5 times the expected incidence.

A hematological and immunologic investigation of some unirradiated disharmonious parabionts was made, with findings of spherocytosis and increased incomplete isohemagglutinin antibodies.[4] This supported the belief that blood incompatibilities had led to an isohemagglutinin hypersensitization with production of hemolytic anemia, as part of the parabiosis intoxication.

MATERIALS AND METHODS

As previously reported,[1] young rats of Slonaker strain, inbred but not genetically pure, were paired by litter mates, sex and weight, anesthetized, and a Bunster-Meyer type of celioanastomosis was performed. After recovery, blood exchange between the partners was determined to be about 0.6 per cent of blood volume per minute, as reported also by

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others.[5] A parabiont group separately prepared also had both adrenals removed either from the left or right partner. Thereafter these animals received 1 per cent saline solution to drink, with separately available drinking water.

Following recovery from the operation or operations, right-hand members were given a single x-ray dose of 400, 800, 1,200, or 1,600 r of total-body irradiation. Left partners were shielded from exposure. At intervals from one hour to twenty-nine days thereafter, animals were killed and complete autopsies performed. Descriptions of the histologic preparations were made without knowledge of the previous experimental manipulations, to avoid bias.

RESULTS

Among thirty-eight normal parabiont pairs irradiated at all dose levels, lesions of parabiosis intoxication were identified in eleven (29 per cent). Fifteen rats among the seventy-six partners (20 per cent) were affected. In the hemiadrenalectomized group 59 of 72 pairs showed parabiosis intoxication lesions (82 per cent), and 84 of 144 rats were involved (58 per cent). Table I summarizes the data, giving the major types of pathologic alteration observed.

TABLE I. INCIDENCE OF ALL PARABIOSIS INTOXICATION LESIONS

	NORMAL			RIGHT ADX*			LEFT ADX*		
	PAIRS	RATS	LESIONS	PAIRS	RATS	LESIONS	PAIRS	RATS	LESIONS
Parabiosis Intoxication	11	15	20	29	39	52	30	45	63
Normal	27	61	—	5	29	—	8	31	—
Total	38	76	20	34	68	52	38	76	63

*Adx means adrenalectomized.

Hemolytic Disease and Polycythemia[4].—During life one rat partner was plethoric, with red ears, paws, and tail, termed for convenience the "red" parabiont. Its partner appeared abnormally anemic, with white extremities, termed the "white" parabiont. At autopsy the "red" rat had congested blood vessels, hepatosplenomegaly, and lymphoid hyperplasia. Microscopically hyperplasia of lymphoid, reticuloendothelial, and plasma cells was found, with tuberculoid granulomas in the spleen (Fig. 1).[6, 7] Erythrophagocytosis was prominent, and in larger vessels, blood cells were abnormally closely packed. Occasionally kidney collecting tubules showed epithelial degeneration with pigment casts typical of hemoglobinuric nephrosis.

In the "white" parabiont splenomegaly was attributable to exaggerated extramedullary hematopoiesis in the red pulp, and hematopoiesis was also unusually prominent in sinusoids of liver, lymph nodes, and adrenal cortex. The bone marrow of "white" rats showed striking myelonecrosis[8] and hyperplasia of hematopoietic stem cells.

While more frequent in hemiadrenalectomized than in normal pairs, no morphologic differences in the severity of lesions were evident between these groups (Table II).

Blood studies showed the "red" rat to be polycythemic and hypertensive, and the "white" rat to be anemic with reticulocytosis and normoblastemia. Hypertrophied or leukocytoid lymphocytes were increased in "red" rats' blood smears.

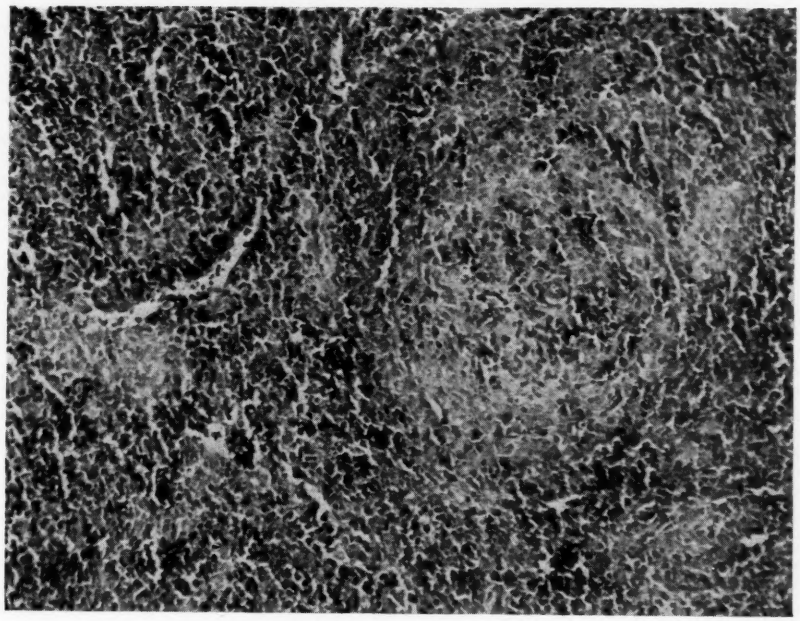
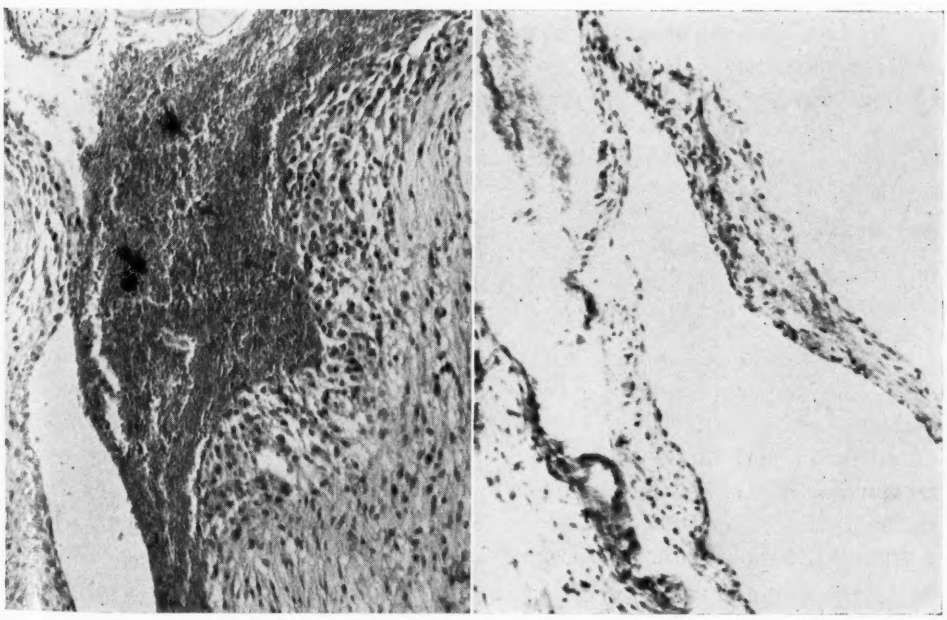


Fig. 1.—Tuberculoid granulomas in the spleen of an adrenalectomized "red" parabiont with hemolytic disease. All slides stained with hematoxylin and eosin. (×125.)



A. *B.*

Fig. 2.—*A*, Mitral endocarditis, with proliferation and palisading of endothelial and stromal cells, from an adrenalectomized parabiont. (×125.) *B*, Normal mitral valve, for comparison, from a normal parabiotic rat. (×125.)

TABLE II. INCIDENCE OF HEMOLYTIC ANEMIA*

	RIGHT†	LEFT	TOTAL
76 Normal parabionts	0	2	2
68 Right† adrenalectomized	9	0	9
76 Left adrenalectomized	3	6	9

*Refers to sensitized "red" parabiont.
†Irradiated parabiont.

Serologic tests were performed with washed rat erythrocytes, incubated with sera from rats with parabiosis intoxication, and the sensitized cells added to serial dilutions of rabbit antisera to rat blood. Increased titers of incomplete hemagglutinins were found in both "red" and "white" partners. Trypsinized erythrocytes showed increased agglutination and hemolysis with sera from animals with parabiosis intoxication, particularly marked with sera of "red" parabionts.

Valvular Endocarditis.—Microscopically the mitral or aortic valve, or both, showed edematous thickenings of the cusps, with myxoid or chromatropic changes of the stroma. Over this area endothelial cells were often relatively increased in size and numbers, and occasionally were palisaded. At times lymphocytes were collected at the valve bases (Fig. 2). Rarely there were additional interstitial perivascular collections of lymphocytes. Similar lesions have been reported in animals after injections of foreign proteins, and have been compared to the cardiac lesions of rheumatic fever[9, 10] or serum sickness[11] in man.

In hemiadrenalectomized pairs the endothelial proliferation, palisading, and intermixture of lymphoid and monocytic cells were both more frequent and enhanced in severity, compared to other parabionts (Table III).

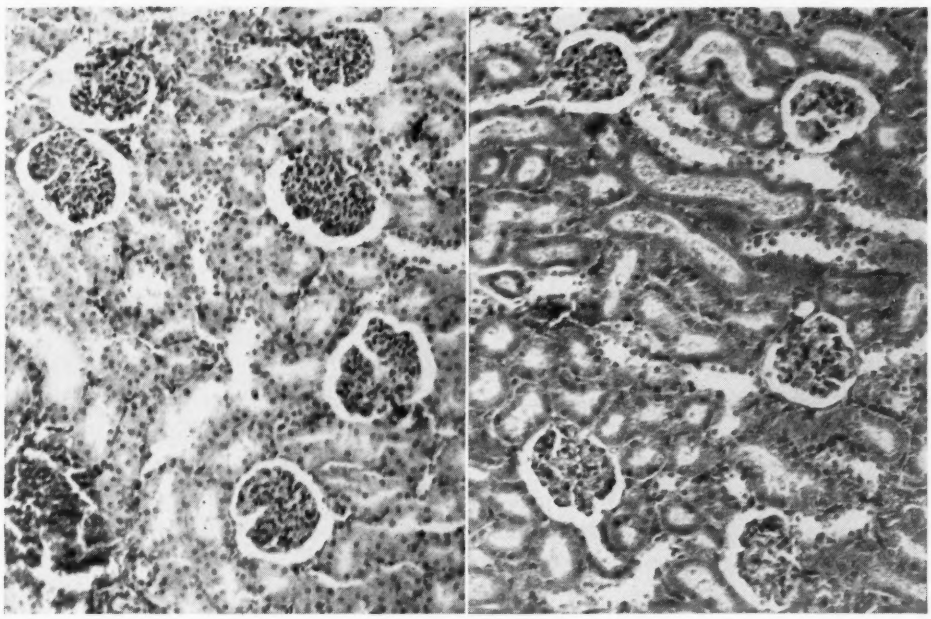
TABLE III. INCIDENCE OF CARDIAC VALVULITIS

	RIGHT*	LEFT	TOTAL
76 Normal parabionts	6	8	14
68 Right* adrenalectomized	17	10	27
76 Left adrenalectomized	12	19	31

*Irradiated parabiont.

Proliferative Glomerulonephritis (Glomerulitis).—In normal parabionts no glomerular changes were observed. With parabiosis intoxication, glomeruli appeared abnormally cellular due to the enlargement and increased numbers of nuclei of endothelial or epithelial cells, usually both (Fig. 3). Basement membranes at times were thickened and appeared hyalinized. Other vessels in the kidney and elsewhere, such as in the pancreas, in a few instances showed a hyaline arteriolar thickening characteristic of arteriolar sclerosis.

Experiments with foreign protein injections,[12-14] including use of anti-kidney sera,[15, 16] have resulted in the production of similar glomerular changes referred to usually either as glomerulonephritis or glomerulitis. Since necrosis, capsular adhesions, and crescent formation were not observed in the relatively short-term experiments described, the microscopic changes do not



A. *B.*

Fig. 3.—*A*, Glomerular proliferative changes in the kidney of an adrenalectomized parabiont. (×150.) *B*, Normal glomeruli, for comparison, from a normal parabiont. (×150.)

Fig. 4.—Necrotizing arteritis and periarteritis observed in the periadrenal connective tissues of a rat from a pair not hemiadrenalectomized. (×125.)

correspond with clinically evident cases of human glomerulonephritis. Hematuria was not sought for in the rats. While more frequently observed, no more severe glomerular lesions were seen in parabiosis intoxication after hemiadrenalectomy than were evident in disharmonious partners with adrenals (Table IV).

TABLE IV. INCIDENCE OF GLOMERULONEPHRITIS

	RIGHT*	LEFT	TOTAL
76 Normal parabionts	1	1	2
68 Right* adrenalectomized	8	8	16
76 Left adrenalectomized	7	17	24

*Irradiated parabiont.

Necrotizing Arteritis.—In a few instances, lesions of medium sized or small arteries occurred in the cervical region or the retroperitoneal connective tissues. The walls of involved arteries were edematous, slightly infiltrated with leukocytes, and with bright red staining material intramurally, considered to be "fibrinoid." Periarterial collections of leukocytes, proliferation of fibroblasts, and thrombosis were present (Fig. 4). The changes resembled those experimentally produced by foreign protein injections,[17, 18] sometimes compared to human periarteritis nodosa.[19] Arteritis did not occur sufficiently frequently to warrant tabulation of its incidence and was not more frequent or severe in hemiadrenalectomized pairs. Joints were not examined for synovial lesions.[20, 21]

Combinations of the individual types of lesions were observed in three of the fifteen animals (20 per cent) with parabiosis intoxication in the group with the normal complement of adrenals. Among comparable hemiadrenalectomized rats, twenty-six of eighty-four (31 per cent) had more than one type of lesion. The most frequent combination was cardiac valvulitis and glomerulonephritis (Table V).

Irradiation, given always to the right-hand parabiont, did not alter the frequency or microscopic appearances of the four types of lesions described previously. Lymphoid tissues in irradiated rats were partly destroyed, as indicated by necrobiosis of lymphocytes. Plasma cells, macrophages, and reticulum cells showed considerably less damage.[22-24] Also the lymphoid and thymic hyperplasias, when present in hemiadrenalectomized pairs, were not usually rendered atrophic by irradiation to the extent observed in normal pairs.

The predominant localization of the pathologic lesions of parabiosis intoxication was in the adrenalectomized partner, whether irradiated or protected (Table VI). Almost twice as many lesions were found in the rat without adrenals as in its partner with intact adrenals, i.e., 76 of 115 lesions (66 per cent). Also the adrenalectomized parabionts had combined lesions in twenty-two of twenty-six such cases (85 per cent). This appeared to indicate the importance of decreased adrenal cortical function both generally in the production and specifically in the localization of lesions in parabiosis intoxication.

TABLE V. INCIDENCE OF COMBINATIONS OF PARABIOSIS INTOXICATION LESIONS

	RIGHT†	LEFT	BILATERAL	TOTAL
76 Normal Parabionts:				
(a) Hemolytic disease and (b) cardiac valvulitis	0	2	0	3 2
(a) and (c) glomerulonephritis	0	0	0	0
(b) and (c)	0	1	0	1
(a), (b), and (c)	0	0	0	0
68 Right† Adrenalectomized:				10*
(a) and (b)	3	0	0	3
(a) and (c)	1	0	0	1
(b) and (c)	4	1	1*	6
(a), (b), and (c)	1*	0	0	1
76 Left Adrenalectomized:				16*
(a) and (b)	2	2	0	4
(a) and (c)	0	1	0	1
(b) and (c)	1	8	1*	10
(a), (b), and (c)	0	2*	0	2

(a) = Hemolytic disease; (b) = cardiac valvulitis; (c) = glomerulonephritis.
*Pair recorded in two categories.
†Irradiated parabiont.

TABLE VI. LOCALIZATION OF PARABIOSIS INTOXICATION LESIONS

	TOTAL LESIONS		COMBINATIONS	
	RIGHT*	LEFT	RIGHT†	LEFT
68 Right Adrenalectomized	34	18	9	1
76 Left Adrenalectomized	22	42	3	13

*Irradiated parabiont.

DISCUSSION

Experiments have been reported in which disharmonious parabiotic rats developed microscopic cardiovascular, glomerular, and gross and microscopic reticuloendothelial and hematological abnormalities. The pathologic changes were considerably increased in frequency and sometimes in severity, particularly localized to one partner following the removal of its adrenals. X-ray exposures had no apparent effect upon suppressing, enhancing, or localizing these lesions.

What does parabiosis intoxication represent? According to a recent review,[25] it "seems to be fundamentally a result of constitutional incompatibility between the two partners, complicated by infection, inanition, and other factors which either accentuate or mask the primary symptoms." From the pathologic, serologic, and hematological studies reported, the evidence collected points to antigen-antibody reactions between parabionts as the basis of parabiosis intoxication. In the syndrome of hemolytic anemia and polycythemia, sensitization to incompatible erythrocytes occurred. From the cardiac, renal, and arterial lesions, hypersensitivity to soluble protein antigens is inferred.

When rats are injected with rabbit antisera to rat kidney, a chronic renal disease is produced with glomerular lesions like those reported previously.[15] In rabbits, injections of horse serum or other proteins have repeatedly been reported to result in acute diffuse glomerulonephritis, cardiac lesions of rheumatic type, and arterial lesions resembling human periarteritis nodosa.[9-11, 13, 14, 16-21]

Studies by quantitative immunologic techniques leave little doubt that such lesions in rabbits are determined by antigen-antibody reactions in the presence of circulating antigen.[26] The experimental lesions in the disharmonious rat parabionts are considered to be analogous hypersensitivity reactions. Parabiosis intoxication, like the biologic basis of individuality, has many facets,[27] but its morphologic manifestations are chiefly those of antigen-antibody reactions. Short of the demonstration in rats of antigens and antibodies, however, proof is not conclusive.

In "red" and "white" rats with parabiosis intoxication, combinations with cardiovascular and renal lesions have been noted particularly in the hypertensive "red" partner,[28] whose polycythemia appeared responsible for the blood pressure rise.[4] Experimental rat hypertension after unilateral nephrectomy plus desoxycorticosterone acetate (DCA)[29] or renin[30] therapy, or contralateral renal encapsulation[31] is associated with lesions like periarteritis nodosa, and sometimes an endocarditis compared to rheumatic lesions.[29] In these conditions no reason for invoking hypersensitivity is known. Basically the damage is to endothelium, vessel wall collagen, and ground substances.[30] The reactions are nonspecific,[32] and are influenced by various mechanical and chemical factors, including hormones. Hypertension generally exaggerates, and may initiate, cardiac, renal, and vascular lesions of identical type in rats[33] or man.[34] The combination of (a) antigen-antibody reactions, (b) hypertension, and (c) certain chemicals, when employed, including sodium chloride, renin, and desoxycorticosterone acetate (DCA), apparently can increase the incidence and severity of such lesions.[35, 36]

Why did hemiadrenalectomy increase the incidence of parabiosis intoxication? Years ago it was established that adrenalectomy would increase the typhoid agglutinin response of rats, with titers up to three times greater than controls after three weeks.[37] Adrenalectomy also produced an increased sensitivity in rats ordinarily resistant to anaphylactic shock with horse serum.[38, 39] When supported with salt and DCA, adrenalectomized rats showed no significant differences in antibody production.[40]

In mice also adrenalectomy increased anaphylactic sensitivity.[41] In adrenalectomized rabbits sheep hemolysin titers were reported over twice the average of controls,[42] horse serum precipitins were increased greatly,[43] Arthus's phenomenon was more frequently positive,[44] and cardiovascular and renal lesions were increased. Complement fall was more regularly observed.[45] Adrenalectomy of guinea pigs[46] or cats maintained on DCA[47] did not alter their antibody production.

These reports indicate that in rats[37] and rabbits[43] higher antibody titers after adrenalectomy were associated with lymphoid and hematopoietic hyperplasias. A casual relation between a hypertrophied lymphoid system and increased antibody production has been repeatedly suggested.[48] In the hemolytic anemia of parabiosis poisoning, the sensitized "red" rat had lymphoid, reticuloendothelial, and plasma cell hyperplasias with increased circulating leukocytoid (hypertrophied, stress[49]) lymphocytes. These last cells, derived

from lymphocytes, are believed to be transitional stages developing into macrophages.[50, 51] Sensitization to erythrocytes was associated with maximal erythrophagocytosis. Similar phagocytic cells react to and may be responsible for antibody formation after protein injections.[52] Lymphocytes may be regarded as generally too immature and Marschalko-type plasma cells as too senile for much antibody production.[53, 54]

Cortisone and hydrocortisone are the individual adrenal cortical steroids chiefly implicated in altering the development and effects of antigen-antibody reactions. ACTH and cortisone cause destruction of lymphocytes[55, 56] and plasma cells.[57] Cortisone and ACTH interfere with the production of hematopoietic,[58] reticuloendothelial,[59] and lymphocytic cells, so that cortisone normally acts as a brake upon the proliferation of cells implicated in antibody formation.[60] When, however, cortisone is injected into animals previously immunized, its destructive action may release intracellular antibodies already produced.[60, 61]

An equally or more significant effect produced by cortisone is to inhibit substances which increase cell, membrane, and blood vessel permeabilities.[62-64] This action interferes with the union of antigen and antibody in hemolytic anemias.[65] Maintenance of permeabilities causing interference with leukocytic migration and phagocytosis[51, 66] may be partly responsible for ACTH and cortisone suppressing the cardiovascular and renal lesions in rabbits injected with horse serum,[67] and the similar lesions produced in rats with DCA.[68] After adrenalectomy, increased membrane permeabilities would allow a freer diffusion and facilitate the interaction of antigens and antibodies.

The biochemical mechanism by which cortisone produces decreased membrane permeabilities is as yet unsettled, but has been ascribed to the formation of increased amounts of hyaluronidase inhibitors,[69] which interfere with the depolymerization of hyaluronic acid and the appearance of metachromatic compounds.[70] This protective effect on viscous ground substances may be mediated by the stimulation of mast cells to produce antihyaluronidase active lipoproteins.[69] After adrenalectomy normal ground substance viscosity is not maintained and various anaphylactogenic substances such as histamine, hyaluronidase, and necrosin are better able to spread and damage cells.[26, 41, 44, 56, 71]

On the basis of present knowledge, the increased frequency of parabiosis intoxication lesions after hemiadrenalectomy could thus be explained by the sequence of (a) increased formation of autoantibodies, (b) increased permeability, particularly of blood vessel endothelium, due to physicochemical alterations of ground substances, (c) facilitation of antigen-antibody combinations, and (d) enhanced local damaging effects of various substances released by the hypersensitivity reactions. It should be emphasized that this explanation is hypothetical and based partly on concepts not at present firmly established. Note that the hemolytic anemia had a cellular antigen, i.e., erythrocytes coated with antibody protein, which altered cellular reactions toward maximal erythrophagocytosis.

The cardiovascular and renal lesions, more likely due to soluble protein antigens, developed without the apparent necessity for much phagocytic

activity. These histopathologic cardiorenal-vascular manifestations were nonspecific,[32] similar to a number of nonallergic conditions such as those produced by nephrectomy, salt, and DCA. The lesions were adversely influenced by the abnormal hemodynamics and hypertension observed, and are ameliorated by cortisone, according to Hall and Hall.[72]

It is significant that nearly two-thirds of parabiosis intoxication lesions in hemiadrenalectomized rats occurred in the partner without adrenals, and consequently relatively more deficient in its hormones. Sites for antigen-antibody reactions were evidently more favorable and resulting tissue damage was more often evident pathologically in conditions of homolateral adrenal hypofunction. The same explanation is suggested for the greater frequency of combined lesions in adrenalectomized partners.

Although irradiation had important and sometimes lethal effects on these parabionts, it is notable that it did not apparently alter the development or localization of hypersensitivity reactions already underway. Whether this was due to the radioresistance of reticulum cells,[23] the massive lymphoid and plasma cell hyperplasias already developed, or to an inadequate sensitivity of histopathologic observations is uncertain.

The cardiovascular and renal lesions found are comparable to those described in "diseases of adaptation" by Selye,[73] and ascribed by him to increased pituitary-adrenal secretory activity. He considered over-secretion of "salt-active corticoids," or DCA therapy, to be responsible. The enhanced incidence after hemiadrenalectomy indicated, however, conversely that in this experiment it was decreased adrenal function that favored the development of lesions like those found in some "collagen diseases." One wonders whether the therapeutic effects of cortisone in human "collagen diseases" do not perhaps indicate that adrenal hypofunction may be a significant factor in their pathogenesis also.

SUMMARY

Hemiadrenalectomy of parabiotic rats increased the incidence of pathologic evidences of parabiosis intoxication from 29 to 82 per cent. Acquired hemolytic anemia, endocarditis, glomerulonephritis, and occasionally arteritis, were observed. Lesions occurred twice as frequently in the adrenalectomized rat as in its parabiont. Combinations of lesions also occurred more frequently in the adrenalectomized parabionts. The factors responsible for the increase of these lesions are considered to be an increased formation of autoantibodies, increased blood vessel and cell permeabilities due to altered ground substances, facilitation of antigen-antibody combinations, and enhanced local cell damage produced by the substances thus released. Irradiation of one parabiont rat did not apparently alter the development or localization of the lesions.

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THE OCCURRENCE OF A PAS-POSITIVE PROTEIN FRACTION IN PAPER ELECTROPHORESIS OF SERUM IN MYELOMATOSIS

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With the Technical Assistance of May Yamaoka, M.T. (A.S.C.P.)**

MULTIPLE myeloma or myelomatosis is a neoplastic disease of the reticuloendothelial system characterized by diffuse or local proliferation of atypical and immature cells generally resembling plasma cells. The disease presents two peculiarities of interest: the occurrence of unusual proteins in the blood and urine, and the finding of disseminated deposits of atypical amyloid or paramyloid in a significant proportion of cases.

The altered serum protein pattern lends itself readily to analysis by electrophoretic methods. Using the moving boundary technique of Tiselius,[22] this abnormality has been extensively studied and documented by Longsworth and associates[17] and by Gutman and others.[1, 9] Recently Griffiths and Brews[7] have utilized filter paper electrophoresis in a further analysis of the serum proteins in this disease.

Filter paper electrophoresis (or ionophoresis) first described by Durrum[3] has been enthusiastically applied to the analysis of serum proteins by European investigators.[13, 14] The procedure presents several advantages over the Tiselius method, not the least of which are simplicity and economy. Furthermore, it is possible to make duplicate strips and subject the physically separated fractions to various tinctorial, chemical, or physical maneuvers for the purpose of comparison. By increasing the time or voltage it is also possible to increase the separation of the serum protein components, within limits.

In the present investigation the protein patterns in a further group of twelve cases of myelomatosis have been analyzed. In view of the occurrence of polysaccharide in fixed amyloid as shown by Hass,[10] the duplicate filter paper strips have been subjected to a modified McManus-Hotchkiss PAS stain in order to determine whether an increase in polysaccharide was also present in the abnormal circulating globulin. A third strip in each instance was stained with aqueous crystal violet.

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METHODS

Electrophoresis.—The electrophoretic separation was performed at a pH of 8.6 in a barbiturate-HCl buffer having an ionic strength of 0.05. The buffer was prepared as follows:

1/10 M. hydrochloric acid	129 ml.
1/10 M. Veronal sodium (Sodium barbital)	871 ml.
Distilled water	1,000 ml.

Filter paper electrophoresis was carried out by the method and on the apparatus of Köiw and colleagues.[14] Whatman 3 mm. filter paper strips, 40 by 5 cm., were used. Using a micropipette, 50 lambda (0.05 ml.) of native serum was carefully applied in a transverse line 9 cm. from the cathode end of the filter paper previously moistened in buffer. The paper strips were then mounted and sealed between heavy glass plates. Several strips could be mounted, separated by strips of thin plastic fabric. The whole assembly was mounted abridging two plastic reservoirs, each containing equal amounts of the buffer and in each of which was immersed a platinum wire electrode. The electrodes were separated from the ends of the strips by baffles and were attached to a power source set to deliver 100 volts and 1 to 10 Ma. The gamma globulin tends to end up slightly toward the cathode as the result of endosmosis.

The apparatus was operated in a draft-free room at room temperature. Variation in the temperature produced no appreciable improvement in separations and when the apparatus was operated at 2° to 4° C. there was no more effective separation than at room temperature. The proteins were exposed to the current for twenty hours, after which the strips were removed, dried at 25° to 50° C., and stained.

Staining.—A. Three staining methods were assessed for demonstration of the protein patterns:

1. Bromphenol blue[16]
2. Amidoschwarz 12B (Naphthalene black)[4]
3. Azocarmine B.[20]

In accordance with the experience of Franglen[5] and others, the two latter proved superior to the bromphenol blue. The protein patterns in the illustrations were stained with amidoschwarz 12B. A normal strip is illustrated in Fig. 1.

B. The modified McManus-Hotchkiss method as described by Köiw and associates[15] was used for the demonstration of polysaccharide-conjugated protein. This method was applied to a duplicate strip run concurrently with the strip used to show the primary protein pattern. The color is not permanent and on exposure to air the unstained portion of the strips turned pink after several hours. The photographs were made using freshly stained strips.

C. Crystal violet was applied to a third strip in a number of instances. The method used was as follows:

Staining solution:

Crystal violet—14 per cent (aqueous solution)	10 ml.
Distilled water	300 ml.
Hydrochloric acid	1 ml.

Procedure.—The filter paper strips were immersed in the staining solution for one minute and rinsed in tap water. Rinsing took several hours.

Scanning.—The electrophotometric model of Griffiths[6] has been modified. The strips were mounted between two glass slides which were fixed to a typewriter carriage. The space bar moved the strips at 1/10 inch intervals over a transverse slit centered on a constant low voltage light. The transmitted light fell on a barrier-type photoelectric cell and the intensity was recorded on a galvanometer. The percentage transmittance was then recorded on 1/10 inch graph paper providing visible correlation between the protein pattern and the curve.

Material.—Native serum from twelve proved cases of multiple myeloma was subjected to electrophoresis and staining. These cases were consecutive. No attempt at case-finding was made, the cases occurring over a period of a few months. The cases all presented unequivocal clinical and laboratory evidence of the disease. Of the twelve patients studied, five died during the course of this investigation and there were autopsies on four. The diagnosis of myelomatosis was confirmed in each instance. (It is not the purpose of this presentation to review the clinical and pathologic features which have been covered completely in excellent current reviews.[21, 23])

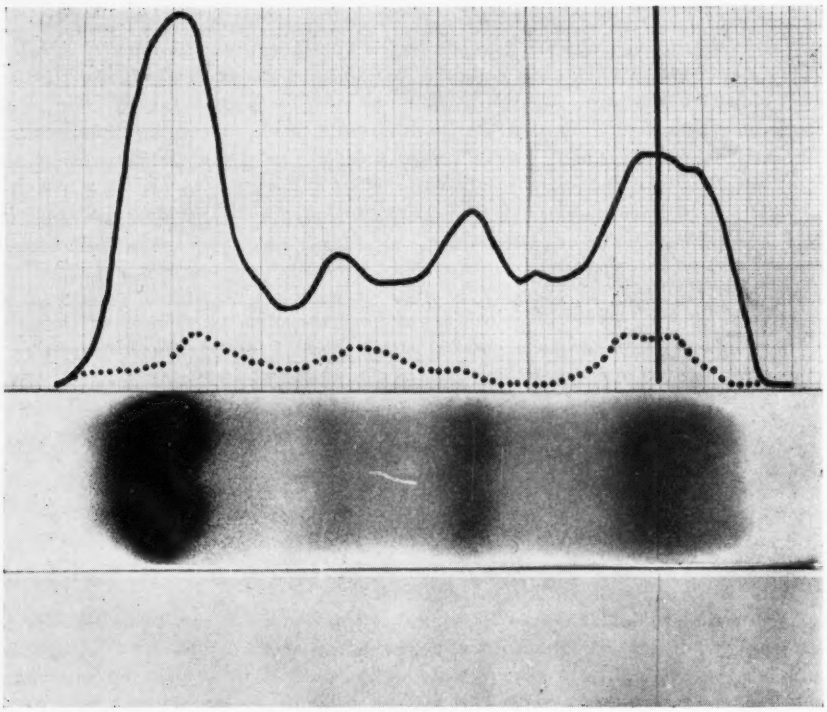

Fig. 1.—This shows a normal protein electrophoretic pattern. The upper strip is stained with amidoschwarz 12B. The heavy line of the graph corresponds with the percentage transmittance of corresponding parts of the stained filter paper strip. From left to right the major protein fractions are albumin, alpha$_2$ globulin, beta globulin, and gamma globulin. The alpha$_1$ globulin is at the trailing edge of the albumin.

The lower strip has been treated by the McManus-Hotchkiss reaction. The faint pink bands associated with each globulin are not apparent in the photograph. The dotted line represents the transmittance of this strip.

RESULTS

1. *Position and Quantity of Myeloma Globulin.*—a. In six cases the abnormal globulin was in the neighborhood of the gamma position. In four of these the abnormal globulin obliterated the locus so that normal gamma globulin could not be distinguished. The protein pattern of one of these is illustrated in Fig. 2. In the other two cases an abnormal globulin only partially covered the gamma position and normal gamma globulin could be seen emerging on the cathode side. In one of these the total globulin was not increased, although a distinct mass of abnormal protein was present at the gamma position. This is illustrated in Fig. 3. The other five cases in this group showed a marked increase in the abnormal globulin.

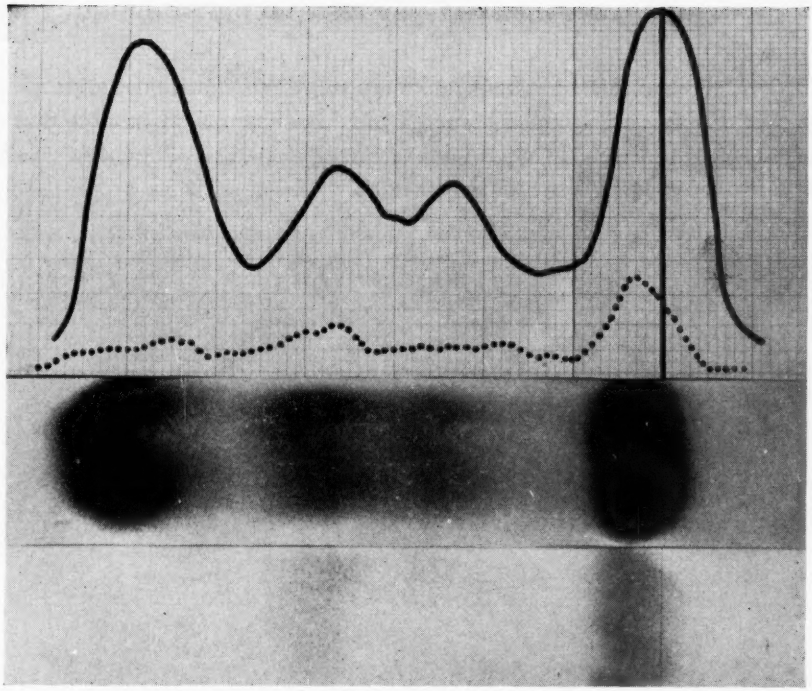

Fig. 2.—This pattern reveals a large concentration of abnormal globulin slightly to the left of the normal gamma globulin position. There is a corresponding PAS-positive band in the lower strip. There is also a faint polysaccharide increase corresponding to increased density in the alpha$_2$ position. These patterns should be compared with the cryoglobulin in Fig. 6 from the same case.

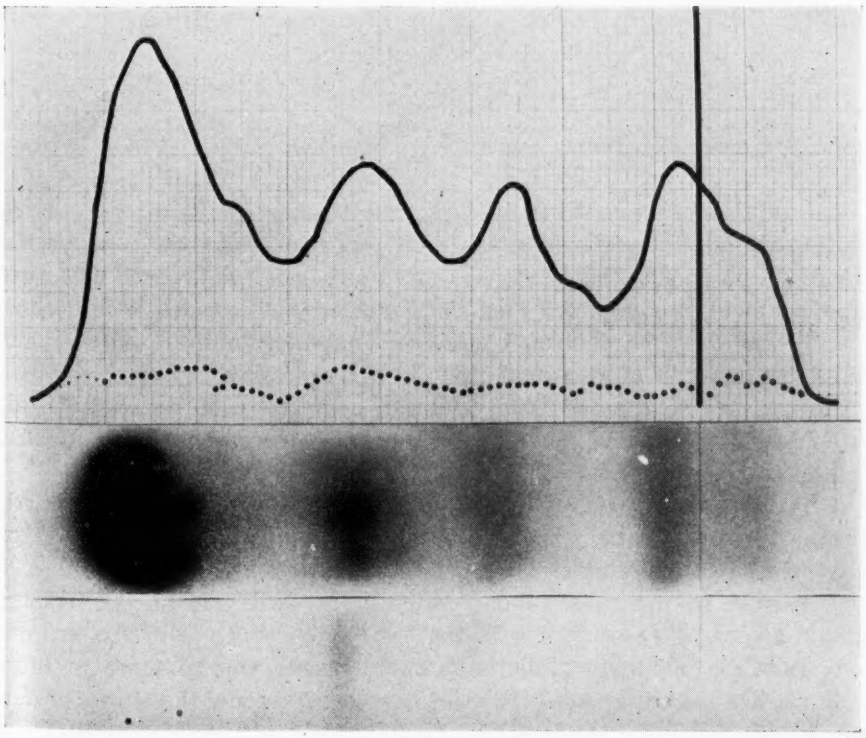

Fig. 3.—This case shows an increase in the globulin component at the alpha$_2$ position and an abnormal myeloma globulin overlapping the gamma position to the left of the starting line. This case is unusual in that there is no polysaccharide corresponding to the myeloma globulin. A distinct band is apparent in the lower strip at the alpha$_2$ position, however.

b. In four cases the myeloma lay between the beta and gamma positions. The degree of migration varied from 1.1 to 1.5 inches with normal beta globulin migration varying from 2.1 to 2.4 inches in this group. A characteristic pattern from this group is illustrated in Fig. 4.

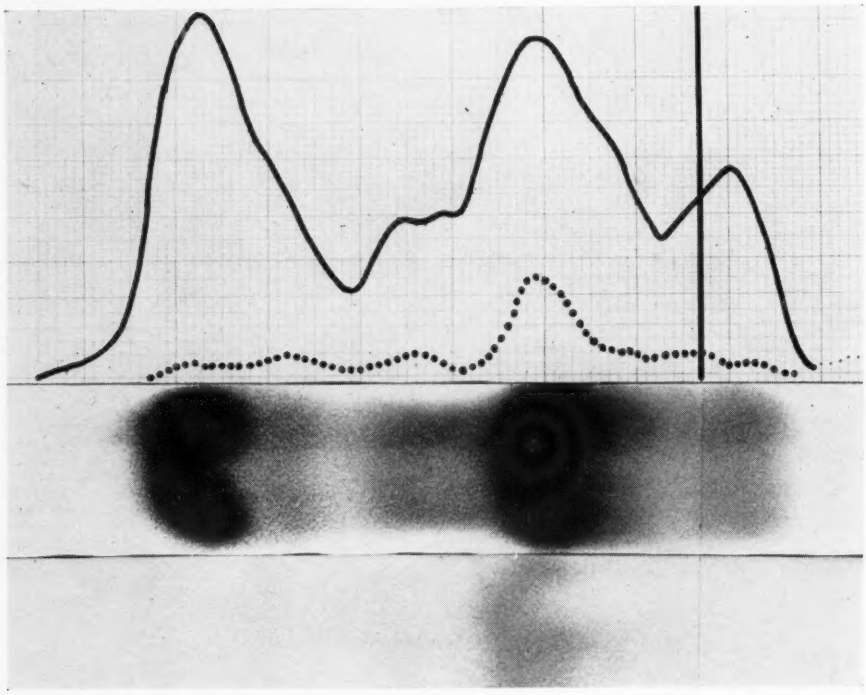

Fig. 4.—This case shows an excess of globulin slightly to the right of the average beta position. The lower strip reveals an increase in PAS-positive material in a corresponding position.

One of these cases also showed no quantitative increase in the myeloma globulin, although there was a distinct abnormal component between beta and gamma positions. This case was in a 57-year-old white man who pursued a rapidly downhill course and died shortly after the diagnosis was established.

c. In one case the abnormal globulin completely obliterated the beta position. The quantity of abnormal globulin was excessive.

d. In one case the abnormal globulin completely obliterated the alpha$_2$ position. The quantity of globulin was markedly increased. The pattern from this fatal case is illustrated in Fig. 5.

2. *Changes in Normal Globulins.*—There were a number of associated and significant changes in the normal globulins, as well as those described previously. These are as follows:

a. Absent or deficient gamma globulin in three cases. An example is shown in Fig. 5.

b. Increase in alpha$_2$ globulin in three cases. One of these is shown in Fig. 3. It has been suggested that this change represents the effects of chronic renal tubular disease, a frequent complication of myelomatosis. Similar changes have been encountered in nephrotic syndromes due to other causes.

c. Decrease in alpha$_2$ globulin, which occurred in one case.

d. Absence of beta globulin which occurred in one case, illustrated in Fig. 5. This was associated with a concurrent absence of gamma globulin, as has been previously noted.

e. Decrease in albumin. This was apparent in five cases, three of them associated with an increase in the alpha$_2$ fraction and being possibly related to chronic renal tubular disease as has been noted previously.

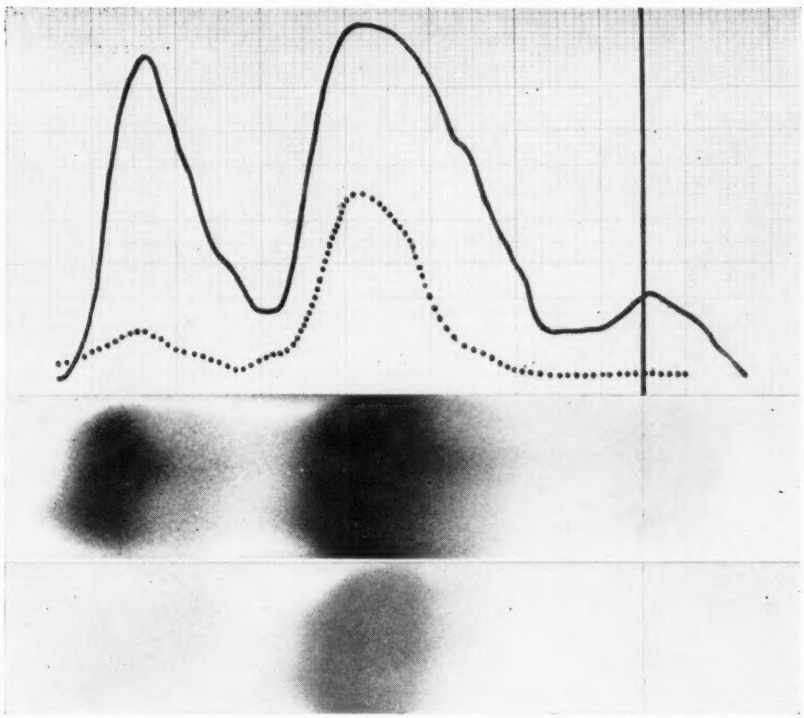

Fig. 5.—This pattern shows a marked aggregation of globulin in the alpha position with a corresponding increase in the polysaccharide in the duplicate strip. The normal pattern is obliterated and there is an absence of beta and gamma globulins and a deficiency of albumin.

3. *Polysaccharide Protein.*—Increase in the polysaccharide fraction corresponding with the abnormal protein as shown by the PAS reaction was noted in ten of the twelve cases. Although normal PAS-positive fractions are seen corresponding to the globulin fractions in normal serum, these are faint. However, in myelomatosis the increases when present were excessive and were associated in each instance with the position of the abnormal globulin.

4. *Crystal Violet Stain.*—In eleven of the twelve cases there was an increased affinity for crystal violet in a position corresponding to the abnormal globulin. The stain was not metachromatic. Normal globulins stained faintly and the albumin fraction only slightly. Since the albumin fraction showed only slight affinity for the crystal violet, the increased intensity of the dye in the myeloma globulin positions was not merely the reflection of an increased

quantity of protein. Globulins themselves, however, may have a nonspecific affinity for crystal violet. The method, in this instance, is not sufficiently sensitive to allow significant analysis quantitatively.

5. *Cryoglobulin.*—In one case, a heavy white precipitate occurred overnight when the native serum was stored at 2° to 4° C. This proved soluble on rewarming. This precipitate was separated by decanting the supernatant serum, dissolved by warming, and subjected to electrophoresis. The results indicate that the cryoglobulin belongs to the abnormal protein component. The pattern of the cryoglobulin electrophoretic pattern illustrated in Fig. 6 may be compared with the serum protein pattern from the same case shown in Fig. 2. A trace of albumin remaining in the dissolved cryoglobulin provides an internal standard.

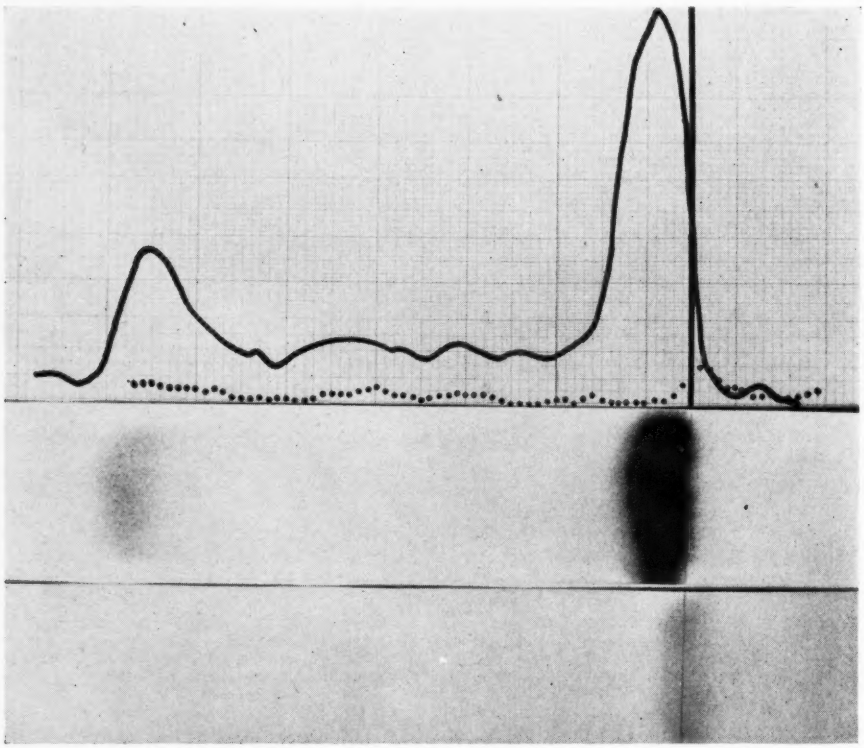

Fig. 6.—This is the electrophoretic pattern of the concentrated cryoglobulins from the case illustrated in Fig. 2. The serum was not washed free and a trace of albumin remains, acting as an internal standard. The PAS-positive band corresponding to the gamma position can be seen distinctly in the lower strip.

DISCUSSION

The findings of previous investigators of the myeloma proteins may be condensed as follows:

1. The abnormality of the serum proteins in myelomatosis involves the globulin fractions.

2. The abnormal protein shows an inconstant position in the protein electrophoretic pattern, being situated anywhere between the gamma and alpha$_1$ positions.

3. The abnormal circulating protein is one of three groups of paraproteins in myelomatosis which represent aberrations from the normal body proteins. The other two are urinary paraprotein (Bence-Jones protein) and tissue paraprotein (paramyloid).[2]

4. Myelomatosis may be subclassified into three[8] or four[7] types in accordance with the position of the myeloma protein in the electrophoretic pattern.

The present investigation has shown that in many instances the abnormal protein does not coincide with the position of the normal serum globulin components, but lies in intermediate positions. This substantiates Apitz's view[2] that these "paraproteins" are abnormal proteins.

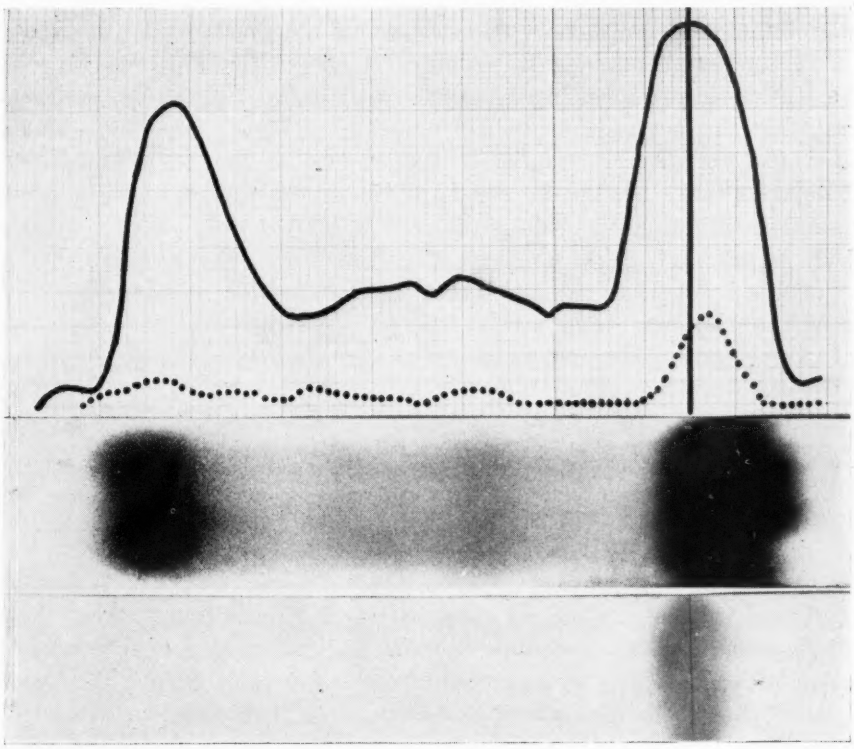

Fig. 7.—This case shows a markedly increased myeloma globulin in the gamma position. There is an associated deficit of albumin. The PAS-positive material in the lower strip occupies only one-half of the total zone occupied by the myeloma globulin.

In view of the long-recognized variations in the appearance of the myeloma cells, it would be surprising to the morphologist if there were not considerable variations in the materials which are presumed to be produced by these cells. The subclassification of myelomas on the basis of cell morphology is in considerable disrepute. This is evidenced by Willis[24] ". . . the nature of the cells . . . is . . . often very doubtful and there is no justification for attempting to subdivide the disease on the grounds of this or that writer's arbitrary identification of the cells." Having discarded the morphologic criteria, there is no reason to subclassify the disease on the basis of a single variable in function.

The starchlike deposition called paramyloid has a frequent but variable occurrence in cases of myelomatosis. In histologic sections this material shows a peculiar affinity for certain dyes, two of the most effective being the McManus-Hotchkiss stain and crystal violet. Hass[10] has previously shown the high content of polysaccharide in fixed amyloid extracted from tissues. It is of interest, therefore, to find that the abnormal circulating globulin in these twelve cases has, with two exceptions, been associated with a coincident increase in PAS-positive material. Admittedly, this may be a purely nonspecific increase common to all globulins. However, Fig. 3 illustrates an instance in which there was no increase in polysaccharide associated with the myeloma globulin. Were the polysaccharide-bearing protein a constant fraction of the total globulin, a definite PAS reaction would be expected in this position. Fig. 7 illustrates an instance in which the PAS-positive material covers only one-half the zone occupied by the myeloma globulin. Both of these instances suggest that the polysaccharide-bearing fraction of the myeloma globulin is variable and not directly related to the amount of the globulin. However, quantitative studies beyond the scope of this paper are required to establish this point. Although the relationship of polysaccharide-bearing protein to fixed tissue paramyloid has not been established, it has been shown that there is a variable increase in the circulating polysaccharide fraction, in the same place as the electrophoretic zone of the abnormal protein. This occurred in ten of twelve cases. The negative cases assume significance since they indicate that the PAS-positive reaction is not a nonspecific quality of all globulins.

In one instance, evidence is also presented to show that a cryoglobulin in a case of myelomatosis has the same electrophoretic pattern as the abnormal globulin from the native serum in the same case. In this case, there was also an increased PAS-positive reaction corresponding to the cryoglobulin peak.

CONCLUSIONS

1. These studies further corroborated Apitz's conclusions[2] that the proteins in myeloma are paraproteins or abnormal proteins. They have shown a variation of the motility in electrophoresis, compatible with a difference in isoelectric point from the normal globulins. The method of zone electrophoresis provided a more precise method of showing this than does the usual salting-out precipitation. Owing to this irregular variation from case to case, it was concluded that subclassification of this disease on the basis of globulin migration was not justifiable.

2. Frequently encountered concurrent alterations in the normal protein components may have been adaptive, as has been previously suggested. Or these changes may have been due to pathologic alterations in renal physiology or to diversion of protein constituents in the body biochemistry.[23] Changes varied from albumin deficit to gamma globulin deficit and may be much more complex than has been previously recognized.

3. Myeloma serum globulins were associated with a coincident increase in PAS-positive material in ten of twelve cases. There was also an increase in

affinity for aqueous crystal violet. These properties are shared by amyloids. The abnormal serum globulins in myeloma may represent a form of circulating paramyloid or paraprotein as has been previously postulated by Apitz.[2]

SUMMARY

The sera of twelve cases of proved myelomatosis were subjected to filter paper electrophoresis. Each showed an abnormal pattern. Ten of the twelve cases showed a coincident increase in PAS-positive material in the same zone as the abnormal protein.

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THE EFFECTS OF WASHING AND OF SERUM ALBUMIN UPON ERYTHROCYTES

III. Upon Cell Volumes

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IT HAS been shown that removal of albumin from the surface of erythrocytes results not only in the cells becoming sphered together with decreases in their diameters when they are spread between glass surfaces,[5] but in loss of their internal movements[6] and in changes in their morphology that indicate conversion of their contents toward an abnormally congealed status. These changes are reversed to normal by addition of serum or of serum albumin to the saline suspension of erythrocytes. The conclusion was drawn that the presence of albumin at the surface of erythrocytes is important to the maintenance of the fluid status of their contents. The present studies were undertaken in search of explanation.

METHODS

Measurement of Erythrocytic Volumes.—Exactness in determination of the differences between erythrocytic volumes under different experimental conditions was more important to these studies than certainty as to absolute values. Attention was therefore centered upon keeping the conditions of measurement uniform rather than upon the problem of real volume. Ponder[12, 13] has discussed the errors inherent in different methods of measuring cell volumes.

All determinations for the present studies (except those described under the heading of Control Studies, part 2) were made in triplicate with van Allen hematocrit tubes and at constant temperature (73° F.). The suspension of erythrocytes to be measured was well agitated before each filling and sealed from the air between fillings. It was pulled to the 10 mark on the scale, followed by admission of the medium under study until the bulb was three-fourths full. The sealed tubes were left upright for one-half to one hour in order to assure equilibrium between medium and cells. They were then spun at 2,155 r.p.m. in an International Centrifuge type SB, head diameter 13.5 cm. The head was allowed to stop without help from the brake. The first spinning was made uniformly for twenty-five minutes. The tubes were read and respun for fifteen minutes. Deviation between the two readings was rare except when attributable to leakage. Any difference not so explained was checked by a third spinning. A reading between individual tubes that differed by more than 1.5 small divisions was considered due to technical error and was discarded. Average of the other two readings was then taken as final. The volumes were calculated from these figures and the companion cell counts. The data are presented in Table I.

Counts of Erythrocytes.—Trenner automatic red blood pipettes and gelatin protected Hayem's solution[3] were used for the samplings. Agitation was procured with an automatic shaker. Levy-Hausser chambers were used for the counts, one side for each pipette. The pipettes were filled in triplicate, but counted in duplicate except where variation between

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samples exceeded 200,000. The samples of whole blood were drawn direct from a finger stick at the times that the studies of volume on whole blood were made. Anticoagulants were not admitted to these. The samples for all other counts were drawn from the experimental suspensions used in the studies of volumes.

Sedimentation Rates of Erythrocytes.—A Wintrobe sedimentation set was used for these measurements. All measurements were made in duplicate and at constant temperature. All tubes for an experiment were filled, let stand, and read at the same time. Readings were made at the end of one, two, and three hours. The color of the columns was also observed. The blood was obtained by either finger stick or venipuncture, without use of anticoagulants. The cells were washed in Locke's solution as described later. They were mixed in the desired media in proportions to give about one-half normal counts. Cell counts were made of each suspension in order that it might be used for calculation of the theoretical sedimentation to be expected on the basis of the control sedimentation obtained in Locke's solution. These studies are described in detail under part 2 of the section headed Control Studies.

TABLE I. ERYTHROCYTIC VOLUMES IN DIFFERENT MEDIA
(Cubic Microns)

DONOR	CONDITIONS	DATE	RBC (×1,000)	VOLUMES WHEN SUSPENDED IN				
				LOCKE'S SOLUTION	DONOR'S SERUM	ALBUMIN IN LOCKE'S PER CENT		
						1.5	4.0	8.0
M.A.G.	*Whole Blood*							
	Heparin	6/11/51	5,175		77			
		5/13/52	5,220		77			
	Oxalate	6/11/51			78			
	Defibrinated Cells							
	Washed	6/11/51	6,075	73	76	82	83	84
		5/13/52	4,475	78	77	86	91	98
	Unwashed	7/11/51	4,480	70	73	74	83	91
E.H.T.	*Whole Blood*							
	Heparin	6/ 4/51	4,780		83			
		6/15/51	4,760		86			
	Oxalate	6/ 4/51			83			
		6/15/51	4,770		83			
	Defibrinated Cells							
	Washed	6/ 4/51	5,750	80	85	88	--	99
		6/15/51	6,247	80	83	88	95	99
	Unwashed	7/ 5/51	4,147	79	81	86	94	99
A.F.G.	*Whole Blood*							
	Heparin	5/15/52	4,100		100			
	Oxalate		4,160		101			
	Defibrinated Cells							
	Washed		6,020	81	85	108	125	133

Collection of Samples.—1. For determinations on whole blood: Blood was drawn directly into the van Allen hematocrit tubes from a finger stick for the determinations using a 1.3 per cent solution of sodium oxalate as anticoagulant. It was drawn into a hypodermic needle from a finger stick for the determinations using heparin and immediately expressed into 1 ml. vials containing standard amounts of the dried anticoagulant. The heparinized blood was drawn to the mark in the hematocrit tubes and the volume determined without addition of a diluent.

2. For all other determinations: Defibrinated blood was used as a starting point for all measurements of volume except those on whole blood. This was obtained by deep finger stick. The blood was allowed to run down the side of a centrifuge tube along which most of the clot collected and stuck. The serum was removed by spinning and saved at 4° C. for the determinations which demanded donor's serum. A portion of the cells was stored at the same temperature for the determinations upon unwashed, defibrinated cells. The remainder of the cells was washed four times in Locke's solution and stored in the last bath. Precautions were taken to exclude any clot with the cells. All refrigerated material was brought to room temperature (73° F.) before use.

Suspensions of Albumin in Locke's Solution.—Eight or ten per cent solutions of crystalline bovine serum albumin (Armour) in Locke's solution were prepared in sufficient quantity for a single experiment. They were made by addition of 8 or 10 Gm. of albumin, respectively, to 100 ml. Locke's solution without correction for the volume added by the albumin. The weaker solutions were prepared from them as needed by appropriate dilutions of aliquots with Locke's solution. The concentrated solutions were prepared in this way because of difficulties encountered with more standard methods of preparation with the materials in question. Locke's solution diluted with distilled water in equivalent proportions to the dilutions caused by the albumin served as checks to the studies of cell volume.* The data from these studies are presented in Table II.

TABLE II. COMPARISON OF ERYTHROCYTIC VOLUMES IN LOCKE'S SOLUTION CONTAINING ALBUMIN WITH THE ERYTHROCYTIC VOLUMES IN LOCKE'S SOLUTION DILUTED WITH WATER IN EQUIVALENT PROPORTIONS

		ERYTHROCYTIC VOLUMES (CUBIC MICRONS) IN:								PERCENTAGE INCREASE COLUMN B COMPARED TO COLUMN A		
				A LOCKE'S SOLUTION DILUTED WITH WATER EQUIVALENT TO THE DILUTIONS WITH ALBUMIN			B LOCKE'S SOLUTION CONTAINING ALBUMIN					
				ALBUMIN EQUIVALENT PERCENTAGE			(PER CENT)			AT ALBUMIN PERCENTAGE		
DONOR	DATE	LOCKE'S SOLUTION	SERUM	1.5	4.0	8.0	1.5	4.0	8.0	1.5	4.0	8.0
M.A.G.	9/15/52	75	79	80	81	80	90	99	107	12.5	22.2	33.8
E.H.T.	9/ 9/52	77	81	75	76	77	81	92	102	8.0	21.1	32.5

Characteristics of Locke's Solution.—Two batches of Locke's solution were used. Lot 2, which was used for all cell volumes on E.H.T. and for the first set of observations on M.A.G., has a freezing point of −0.64 and a pH of 7.62.† Lot 3 was prepared with equal care but unfortunately these physical data were not determined on it. It was used for the second set of volumes of M.A.G. and for those of A.F.G. As the two sets of studies on M.A.G. agree within experimental limits, it is obvious that the physical constants of this lot of Locke's solution must have been quite similar to those of Lot 2. It should perhaps be emphasized again that comparative values, rather than absolute values, are important to these studies, and that comparative values were always obtained by use of the same lot of solution and the same set of conditions for any given set of studies.

Donors.—The donors are the same three women who contributed to the preceding studies in this series.[4-6] Donor M.A.G. is of Italian extraction and has a high preponderance of oval erythrocytes in circulation. Ponder[2] has shown that the volumes and diameters of erythrocytes of persons with this genetic aberrant are smaller than the average for the

*We are grateful to Dr. Ann S. Minot, Vanderbilt Medical School, Nashville, Tenn., for suggesting this method of control.

†These determinations were kindly made for us by Dr. Ralph W. McKee, Physiological Chemistry, University of California Medical School, Los Angeles, formerly a member of this Institute.

remainder of the normal population. The measurements of A.F.G. are greater than normal and the red count is slightly subnormal. The values for E.H.T. are midway between these extremes.

RESULTS

Table I presents the erythrocytic volumes for each individual under the various experimental conditions. The volumes of the cells in serum have the same relationship to the volumes in plasma (1.3 per cent sodium oxalate or heparin) which Ponder obtained with the same anticoagulants. The values in serum are more important to the present studies since they form the base line for the remainder of the studies. It will be seen that the average volumes in serum varied between individuals more than did the average diameters.[5] The erythrocytes had an average diameter of 8.9 μ each, but average volumes of 77, 86, and 100 cu. μ, respectively. Ponder[2] considers the average diameter for human erythrocytes to be 8.5 μ and volume, 87 cu. μ.

While the diameters decreased markedly when the erythrocytes were washed with Locke's solution and suspended between glass,[5] the decreases in volume were slight and disproportionate to the decreases in diameters (Table I). It must be remembered, of course, that the measurements of diameter were made upon washed cells that had become sphered by suspension between glass, while the measurements of volume were upon cells that were similarly washed, to be sure, but not subjected to sphering. Ponder has found that changes in volume of washed erythrocytes from that of unwashed ones depend entirely upon tonicity.[2] The slight hypertonicity of the preparation of Locke's solution used in these experiments probably accounts for such small reductions in volume as do occur.

When the washed cells were returned to serum, or to Locke's solution containing albumin, the volumes increased. When they were suspended in serum their volumes increased to normal in the case of M.A.G. and E.H.T., but only partially so in the case of A.F.G. Their volumes in all of the dilutions of albumin in Locke's solution that were used in these experiments, on the other hand, were greater than in serum, the increase paralleling the increase in albumin. This was true even in a concentration of albumin less than that in blood, namely, 1.5 per cent albumin. No evidences of hemolysis were observed in any of the studies of volume.

Control Studies.—Investigation was instituted to determine if the changes in volume that were obtained under the different concentrations of albumin were real and due to albumin, per se, and actually upon the cells, per se, or if they were possibly artifactual and due to (1) changes in the tonicity of Locke's solution after the additions of albumin, or to (2) excessive retention of fluid along with the centrifuged sediments.

1. The question of effect due to decrease of ionic tonicity of Locke's solution by the addition of albumin was answered in the negative by hematocrit studies in Locke's solution diluted with water in amounts equal to the dilution found with albumin. These were prepared by measuring the volume-increase of a definite volume of Locke's solution when albumin was added according to the method described. An amount of water equal to this increase was added to

an equivalent definite volume of Locke's solution. Aliquots of this diluted Locke's solution were mixed with specific amounts of regular Locke's solution in proportions equal to those used in preparation of the weaker solutions of albumin. Hematocrit determinations were then carried out with the companion solutions of albumin and of diluted Locke's solution.* The results are presented in Table II. The cell volumes did not change significantly in the various dilutions of Locke's solution. They changed in accord with the previous findings in the solutions of albumin. Therefore, the changes in volume of erythrocytes in solutions of albumin were not due simply to hypotonicity produced by the techniques employed in the preparation of the solutions.

2. The possibility that excessive amounts of fluid were retained along with, but essentially separate from, the cell sediments due to changes in viscosity and that they led to erroneously large hematocrit readings, was examined by a combination of sedimentation studies and of differential centrifugation. Sedimentation rates of the cells in the various media were determined and the cells were then spun in the same tubes to constant volume at three different settings of the centrifuge ranging up to 2,155 r.p.m. (head diameter 13.5 cm.). This approach was adopted in the expectation that the rate of fall in hematocrit readings would differ in the varying solutions of albumin if different amounts of water were simply being loosely retained with the sediments due to increasing viscosities. The sedimentation readings obtained in the solutions of albumin were compared to the values calculated (on the basis of the rate of sedimentation in Locke's solution and proportional to the red blood cell counts in each solution) to have been expected. The results are reported in Table III.

TABLE III. SEDIMENTATION RATES, AND EFFECTS OF DIFFERENT RATES OF CENTRIFUGATION UPON ERYTHROCYTES IN ALBUMIN

		SEDIMENTATION IN 3 HOURS			HEMATOCRIT AT DIFFERENT SPEEDS (DIAMETER CENTRIFUGAL HEAD 13.5 CM.)		
		A REAL	B EXPECTED ON BASIS OF RBC	DEVIATION B–A	1,270 r.p.m.	1,800 r.p.m.	2,155 r.p.m.
DONOR	SUSPENSOID	MILLIMETERS					
M.A.G.	Locke's	4.6			18.1	17.6	17.4
	2.0 albumin	4.2	4.1	+0.1	18.9	18.3	18.2
	4.0 albumin	5.0	5.6	–0.6	14.8	14.4	14.4
	8.0 albumin	2.4	3.5	–1.1	21.5	20.7	20.6
E.H.T.	Locke's	5.9 (12.0)			16.8 (4.9)	16.1 (4.9)	16.0 (4.9)
	1.5 albumin	6.0	6.4	–0.4	16.8	16.1	16.0
	4.0 albumin	4.1	5.9	–1.8	18.3	17.3	17.1
	8.0 albumin	(6.6)	(11.3)	(–4.7)	(6.0)	(6.0)	(5.9)

Figures in parentheses were obtained with a different stock supply of erythrocytes from that used for the remaining determinations on E.H.T.

Sedimentation was minimal when the cells were suspended in either Locke's solution alone or in solutions of albumin (Table III). Differences between sedimentation rates could, therefore, be slight at best but, nonetheless, it seems evident that sedimentation is quicker in Locke's solution alone than when albumin is present, and that the decrease with albumin parallels the concentra-

*We are grateful to Dr. Ann S. Minot for suggesting this method of control.

tion. It obviously also parallels the increase in erythrocytic volume obtained synchronous with concentration and could therefore be merely an expression of that. Despite the slowed sedimentation in albumin the changes in hematocrit readings with increasing rates of spinning were essentially the same in all concentrations. It seems, therefore, that neither buoyancy nor retention of extraneous fluid can be brought into force in explanation of the effects of albumin upon sedimentation, hematocrit, and volume of erythrocytes. As far as these experiments permit deduction, therefore, the volumes of erythrocytes washed in Locke's solution increase parallel to the increments of albumin added to the solution.

The columns of cells in the sedimentation tubes showed differences of color after an hour or so of standing that are significant to the deductions to be drawn from the entire series of observations. The columns in Locke's solution alone remained bright, almost brick red. Those in the higher concentrations of albumin, and in serum, became a definite dark blue-red.

In review, then, the volumes of washed erythrocytes increased parallel with the concentrations of albumin, although the diameters remained normal. There were no evidences of hemolysis within the limits of these studies. The sedimentation rates were slightly longer in solutions of albumin than in Locke's solution alone. Columns of cells remained bright red in Locke's solution, but slowly changed to blue-red in solutions of albumin.

DISCUSSION

It has been shown in the two preceding reports[5, 6] of this series of studies that changes result in erythrocytes when they are washed in Locke's solution and suspended between glass which indicate conversion of their contents from the normal fluid to a more jelled or solid status; and that addition of serum albumin to the saline medium causes reappearance of characteristics which indicate recovery of fluid status. The reason for the protection afforded by albumin to erythrocytes against gelling, sphering, and loss of their internal movements under the previously mentioned conditions was not obvious. Ponder[2] found that albumin forms a layer of 50 Å in depth in the ultrastructure of erythrocytes and he theorizes that it may elicit reorientation of molecules which serve to strengthen the ultrastructure, and for lack of which the structure becomes weakened and the cells sphered. It seems probable that this layer either also elicits reorientation of molecules beneath the superstructure into more soluble groupings, or retains sufficient fluid, in some manner, for maintenance of the erythrocytic contents in fluid status. The present findings seem to support the latter assumption.

Ponder found the protective action of albumin against sphering to function in infinitely smaller amounts than those used in the present studies, and without alterations of volume. Like the present data, his studies upon volume were also made upon cells in the unsphered status. It is obvious from the present data, on the other hand, that larger amounts of albumin do elicit appreciable

increases of erythrocytic volumes. It seems probable that changes in volume also occur even with very small amounts of albumin, but that the techniques for measurement are not sufficiently delicate to reveal differences in volume between erythrocytes suspended in albumin-free saline and erythrocytes suspended in saline that contains only very small amounts of albumin.

It seems amazing, on the other hand, that the increases in volume of the erythrocytes which paralleled the increases in albumin occurred without any evidences of hemolysis. Once they returned to normal following additions of albumin to saline suspensions of erythrocytes, the long diameters of the cells remained stationary,[5] in contrast to the stepwise increases in volume found to occur under the same circumstances. These findings are anomalous and would seem to indicate that the erythrocytes balloon without increase in fragility. If albumin penetrates and strengthens the ultrastructure, as postulated by Ponder, it is conceivable that it attracts water, even as it strengthens the ultrastructure, and thereby returns the cellular contents to their normal state of fluidity without injury to the cells in the process.

The fact that the cells remain bright red in the present experiments when packed in Locke's solution but take on blue hues when in solutions of albumin supports the concept that the molecules of hemoglobin in washed cells are in abnormally stabilized form and therefore little subject to the usual oxidation-reduction processes, but reversed to their normal state of flux by addition of albumin to the saline suspension.

It is possible that changes in ionic permeability, or the overwhelming discrepancy between the proportions of albumin and the absence of globulin in these experiments, also account for the volume-diameter behavior of the erythrocytes, but the data do not permit deductions in these respects. It is of interest to note, however, that the change in diameter of the erythrocytes in these globulin-free, but albumin-rich media is greater, and that of the volume less, on the albumin-poor than on the albumin-rich side of the medium.

CONCLUSION

1. Decrease in volume of erythrocytes washed in Locke's solution is slight and out of proportion to the considerable decrease in diameters when the cells are examined between glass.

2. Addition of albumin to saline-washed erythrocytes is followed by increases in volume parallel to the amounts of albumin added, but without increase in the diameter beyond the normal.

3. Columns of saline-washed erythrocytes remain bright red on standing. Columns of saline-washed cells to which albumin is added turn red-blue on standing.

4. The findings are discussed in support of the concept that albumin serves to maintain erythrocytic contents in fluid status and that this status is essential for the normal morphology and the internal movements of erythrocytes.

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ABO GROUPS IN BLOOD PLATELETS

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IN A preliminary communication[1] we reported the results of studies on ABO antigens in blood platelets, and were able to show that human blood platelets were agglutinated by corresponding antisera in the same way as red blood cells are agglutinated by these sera. It was thus possible to establish the existence of four blood groups for platelets, similar to the four blood groups for erythrocytes in the ABO system.

The following is a detailed report on the methods used in this study and the results of further examinations of the antigens in blood platelets.

MATERIALS AND METHODS

All glassware was siliconized before use.[2] Disodium Sequestrene[3] (disodium ethylendiaminotetraacetate dihydrate) was employed as the anticoagulant.

Triton W.R. 1339[2] was used to prevent irreversible clumping of the platelets caused by centrifugation.

A solution composed of disodium Sequestrene 1 per cent, and Triton W.R. 1339 1 per cent, was prepared in sodium chloride 0.7 per cent. Two milliliters of this solution were added to 18 ml. of blood.

Preparation of the Platelet Suspension.—Eighteen milliliters of blood were taken from healthy normal persons in a siliconized syringe containing 2 ml. of the disodium Sequestrene-Triton solution. The blood was immediately transferred into a large centrifuge tube with a flat bottom. (A conical bottom made the resuspension of the platelets difficult.) The blood was centrifuged in a horizontal centrifuge for five to seven minutes at 800 r.p.m. The plasma was pipetted off and centrifuged again for five minutes at 800 r.p.m. in order to remove the remaining red and white blood cells. The supernatant plasma containing a pure suspension of thrombocytes was next centrifuged for ten minutes at 2,000 r.p.m. to obtain a sediment of blood platelets. The plasma was siphoned off and a white pasty mass of platelets remained on the bottom of the tube. Three to five milliliters of saline was added, and, by gently shaking, a suspension of platelets was obtained with an average concentration of 1 to 2 million per cubic millimeter. All centrifugations were performed at room temperature; there was no special advantage in using a cooled centrifuge.[4]

The suspension of platelets contained very few erythrocytes, a rare leukocyte, and often several small clumps of thrombocytes. Refrigeration for one to two hours and occasional slight shaking during this time cause the clumps to disappear.

Preparation of the Sera.—Sera used in agglutination experiments were prepared as follows. Blood was taken from healthy donors using sterile precautions. Sera were inactivated at 56° C. for thirty minutes. Only clear and uncontaminated sera were used. They were titrated for anti-A and anti-B isoagglutinins by the double serial dilution method against freshly prepared red blood cell suspensions.[5] When stored in the freezer (-4° C.), sera maintained their platelet agglutination titer for at least nine weeks.

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EXPERIMENTAL

Agglutination of Platelets.—To 0.5 ml. of test serum in a siliconized tube was added 0.1 to 0.2 ml. of the platelet suspension, depending upon density. The added suspension formed a layer above the serum. The tube was gently shaken to obtain a homogeneous mixture with the antiserum and left in the refrigerator for three hours.

Before reading results, the tubes were gently shaken and examined macroscopically. One drop of the serum-platelet mixture was then withdrawn by a capillary pipette and examined microscopically, at low magnification. In some samples, within three hours macroscopical agglutination could be seen with the unaided eye, and more easily visualized with the magnification loop. Macroscopic readings were repeated every three hours up to twenty-four hours after gently tapping the tubes, and then microscopic readings were performed.

Eighty-five samples of platelets were examined. Platelet suspensions in autoserum, homologous serum, AB serum, and normal saline served as controls. Each sample of platelets was examined against an average of six different antisera with titers ranging between 1:16 and 1:256. Sera with isoagglutinin titers lower than 1:16 gave indistinct results and were therefore not reliable.

The samples examined belonged to the following blood groups: A, 30; B, 24; O, 25; and AB 6.

None of the platelet samples was agglutinated by autoserum, homologous serum, or AB serum. No signs of agglutination were found in platelet suspensions in normal saline. The results of agglutination are summarized in Table I.

TABLE I. RESULTS OF PLATELET AGGLUTINATION WITH A AND B SERA

PLATELETS FROM DONOR OF BLOOD GROUP	A SERUM	B SERUM	O SERUM	AB SERUM	AUTO-SERUM	NORMAL SALINE
A	−	+	+	−	−	−
B	+	−	+	−	−	−
O	−	−	−	−	−	−
AB	+	+	+	−	−	−

Blood group typing of the red blood cells with A and B sera was carried out on all blood samples drawn for the platelet antigen studies. The results showed exact correspondence between the erythrocyte and thrombocyte groups. In not one sample was a deviation observed.

Adsorption of Isoagglutinins by Erythrocytes.—Freshly inactivated undiluted sera were adsorbed twice by adding equal volumes of packed red cells which were washed three times. The adsorption was carried out in the refrigerator for one hour. During this time the serum-cell mixture was shaken every ten to fifteen minutes.[7]

Thirteen different sera were adsorbed by red blood cells and examined for their agglutinating power against thrombocytes. In all these experiments the added platelets remained in a homogeneous suspension. No signs of agglutination were observed.

Adsorption of Isoagglutinins by Thrombocytes.—A platelet concentrate obtained from a large amount of blood (40 to 50 ml.) was resuspended in 0.5 ml. of normal saline. Undiluted sera, or sera diluted with normal saline in a proportion of 1:1, were used in the adsorption experiments. The concentrated platelet suspension was added to 0.5 ml. of antiserum and left for three hours in the refrigerator. The platelet-serum mixture was shaken every fifteen minutes, and after three hours, centrifuged at 2,000 r.p.m. for ten minutes. After the first adsorption the isoagglutinin titer of the sera was markedly reduced. Complete adsorption of isoagglutinins was obtained by a repeated treatment with a platelet concentrate. The same nonadsorbed serum to which 0.5 ml. of normal saline was added was used as a control.

Adsorbed and unadsorbed sera were examined against corresponding red blood cells and no agglutination of the erythrocytes was found in the sera twice adsorbed by platelet concentrates.

Twelve different sera of A, B, and O groups were examined. When A or B sera were adsorbed with platelet concentrates of the corresponding group, no isoagglutinins for red blood cells remained in these sera. When O serum was adsorbed by A or B platelets only the corresponding isoagglutinin was exhausted, while the titer of the other agglutinin remained unchanged. When O sera were adsorbed by AB platelets, adsorption of both anti-A and anti-B isoagglutinins was obtained.

Inhibition of Isoagglutinins by Saliva.—Saliva from A and B secretors was collected in sterile test tubes, boiled for ten minutes, centrifuged, and the supernatant diluted 1:10 with normal saline.

The saliva was then titrated for inhibitory activity. Ten different sera, with titers ranging from 1:64 to 1:256, were diluted in normal saline (1:1). To five-tenths milliliter of the sera was added 0.1 to 0.3 ml. of the saliva, depending upon its inhibitory activity. The saliva-serum mixture was well shaken and left at room temperature for one hour. To the sera treated by saliva, suspensions of red blood cells and platelets of the corresponding blood group were added. The same untreated sera, to which saline was added instead of saliva to achieve equal dilution of the treated sera, served as controls.

All sera in which isoagglutinins were inhibited by saliva did not agglutinate red blood cells or platelets. In the control series agglutination proceeded as usual for both red blood cells and blood platelets.

Neutralization of Isoagglutinins by A and B Substances.—Preparations of the specific polysaccharide blood group A (reagent) and blood group B (reagent) were added to antisera in an approximate proportion of 1:10. After shaking, the sera were examined against corresponding red blood cells to establish neutralization of isoagglutinins. To five-tenths milliliter of neutralized serum 0.1 ml. of a fresh suspension of platelets was added and left in the refrigerator. Fifteen different sera neutralized by A and B substances were examined. No signs of agglutination of platelets were found when examined after three hours, six hours, and nine hours. Control examinations of the same sera not treated by the A and B substances showed the usual results of platelet agglutination.

Isoagglutinin Titer and Intensity of Platelet Agglutination.—The relationship between isoagglutinin titer of sera and the intensity of platelet agglutination was studied in thirty-five different sera. As may be seen in Table II, high titered sera of 1:64 and above gave a more distinct agglutination in a shorter period of time.

TABLE II. ISOAGGLUTININ TITER AND INTENSITY OF PLATELET AGGLUTINATION

TITER OF SERUM	3 HR.	6 HR.	9 HR.	12 HR.
1:128	++	+++	++++	++++
1: 64	++	+++	++++	++++
1: 32	+	++	++	+++
1: 16	–	±	+	+
1: 8	–	–	–	–

When sera with a titer of 1:64 and above were used, agglutinates were larger and the number of nonagglutinated platelets in a microscopic field was much smaller than when sera with titers of 1:32 or below were employed.

Similar results of platelet agglutination were obtained when titrated in saline dilutions of sera, or in undiluted sera with a corresponding isoagglutinin titer.

Influence of Temperature on Agglutination of Platelets.—The first experiments on platelet agglutination by antisera were carried out at room temperature. Results of agglutination were read microscopically after two to three hours. Later on, the differences in agglutination at temperatures of 4° to 6° C., 20° C., and 37° C. were studied. Twelve samples of platelets were examined, each with four different antisera. Comparative readings of the results obtained with the same antiserum at the three different temperatures showed that at 4° to 6° C. agglutination proceeded more rapidly, agglutinates were larger, and the number of nonagglutinated platelets was smaller than at 20° and 37° C.

Influence of Storage of Platelets on Their Agglutinability and Morphology.—Ten specimens of platelets were kept in the refrigerator at +4° C. and examined daily for their agglutinability with the same antisera. As may be seen in Table III, a distinct decrease in the agglutinability of platelets occurred during the first twenty-four hours of storage. When examined on three consecutive days of storage the suspensions showed a slow and continuous decrease in platelet agglutinability by antisera. On the sixth to the seventh day of storage, platelet suspensions tended to spontaneous agglutination. Most of the suspensions were found, at that time, to be contaminated.

TABLE III. CHANGES IN PLATELET AGGLUTINABILITY ON STORAGE AT 4° C.

	3 HR.	6 HR.	9 HR.
1 day	++++	++++	++++
2 day	++	+++	+++
3 day	+	++	+++
4 day	±	+	+
5 day	±	+	+

It may be assumed that the platelet antigens became less and less active during storage, a process similar to the loss of antigenicity of the red blood cells when stored in saline.

Morphologic changes of the platelets were studied during storage, to learn whether a correlation existed between loss of agglutination and morphology. Suspensions of platelets were examined daily by the phase contrast microscope.

In fresh suspensions the platelets were polygonous, many possessed spicules, and there were very few "giant platelets." Most platelets were approximately the same size; about 10 to 20 per cent were smaller than the rest. On the second day of storage the platelets became swollen, rounded up, and showed a granular structure. The "giant forms" increased in number. This process of rounding up and increasing in size progressed until about the fourth to the fifth day of storage when nearly all platelets showed a fine reticular or granular structure.

COMMENT

As early as 1905 Marino[8] studied the antigenicity of blood platelets. Toda[9] in 1923 tried to investigate the antigens of platelets by using human antisera. He did not observe agglutination of platelets in the limited number of his experiments. Stefanini and associates[10-14] studied the antigens of platelets in patients with idiopathic thrombocytopenic purpura, treated with repeated transfusions of platelets. By the use of "immune" plasmas from these patients the authors were able to distinguish six serologically different platelet types. They also reported on the probable existence of four groups of platelets, detected by the use of naturally occurring agglutinins in platelets.[14] Harrington and co-workers[15] established the existence of eight serologically different types of human platelets by using immune antiplatelet sera from human beings and animals.

In our studies eighty-five different samples of platelet suspensions in normal saline were examined as to their antigenic structure by the use of inactivated human antisera. At first, platelets of a known blood type were used in the agglutination experiments. In the second part of this study, platelet suspensions were examined first, and results were then confirmed by red blood cell typing of the same blood samples. Results were found to be identical.

Stefanini, Dameshek, and Adelson[11] have found that repeated transfusions of platelets become progressively less effective and that the survival time of the transfused platelets was shortened. Van Creveld and co-workers[16] reported on nine cases of thrombocytopenia and thrombopathia treated by repeated platelet transfusions. They observed a steady increase in the number of platelets and the patients benefited from the repeated transfusions. These authors stated that as the antigenic structure of the thrombocytes was unknown, they always used blood from the same ABO and Rh group as those of the patient.

The establishment of blood groups for thrombocytes implies the use of only compatible platelets for transfusions on the same lines as for whole blood.

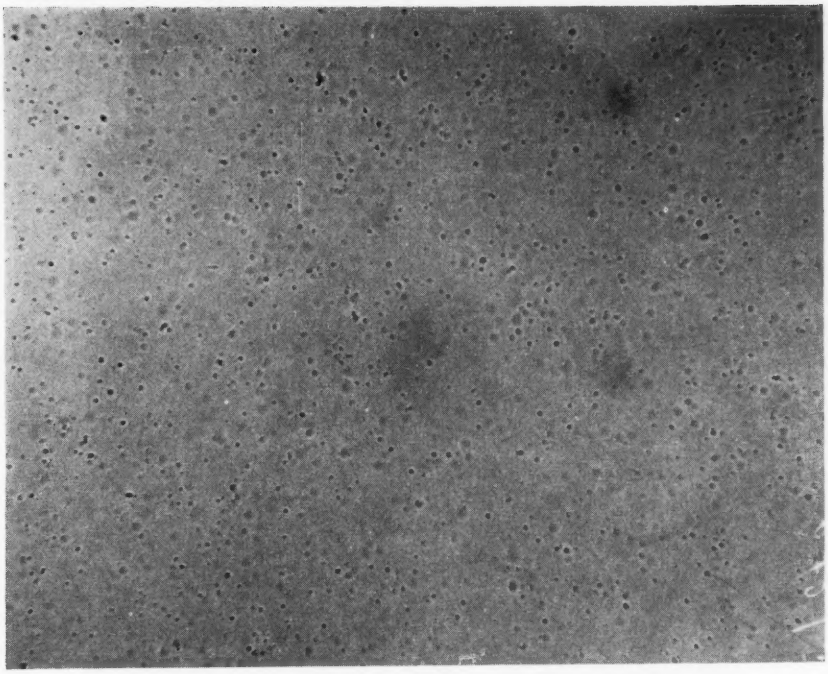

Fig. 1.—Platelet suspension in saline. (Magnification ×150.)

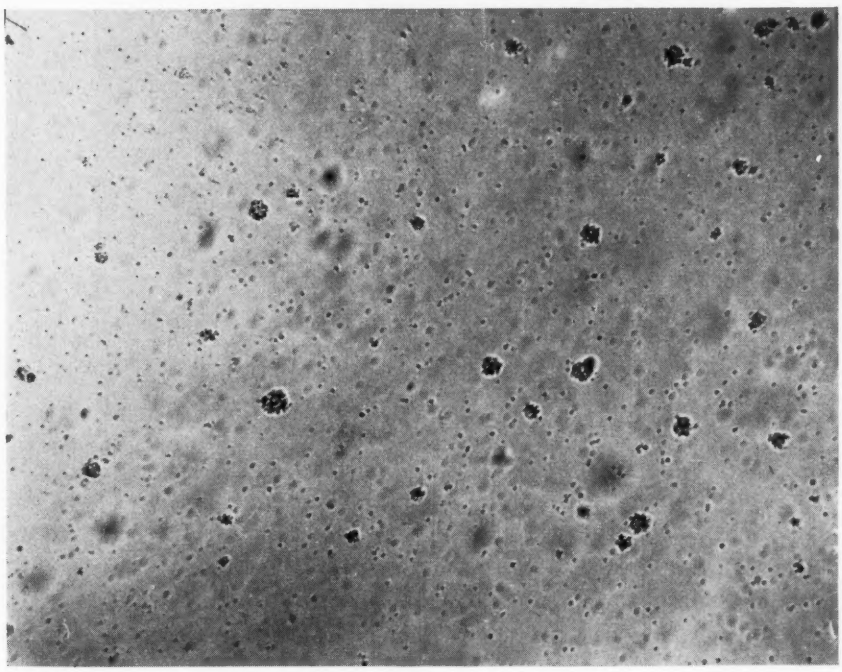

Fig. 2.—Agglutination of platelets by antiserum after three hours. (Magnification ×150.)

In our studies identity of A and B antigens in red blood cells and blood platelets was proved by adsorption experiments. When isoagglutinins were adsorbed by red blood cells, the adsorbed sera did not agglutinate thrombocytes. On the other hand, when the same antisera were adsorbed by large amounts of platelet concentrates, the isoagglutinins were exhausted, so that these sera did not agglutinate red blood cells. Further proof of the identity of the A and B antigens in erythrocytes and in thrombocytes was obtained through inhibition experiments of isoagglutinins by saliva from secretors, and by neutralization of isoagglutinins with A and B blood group substances.

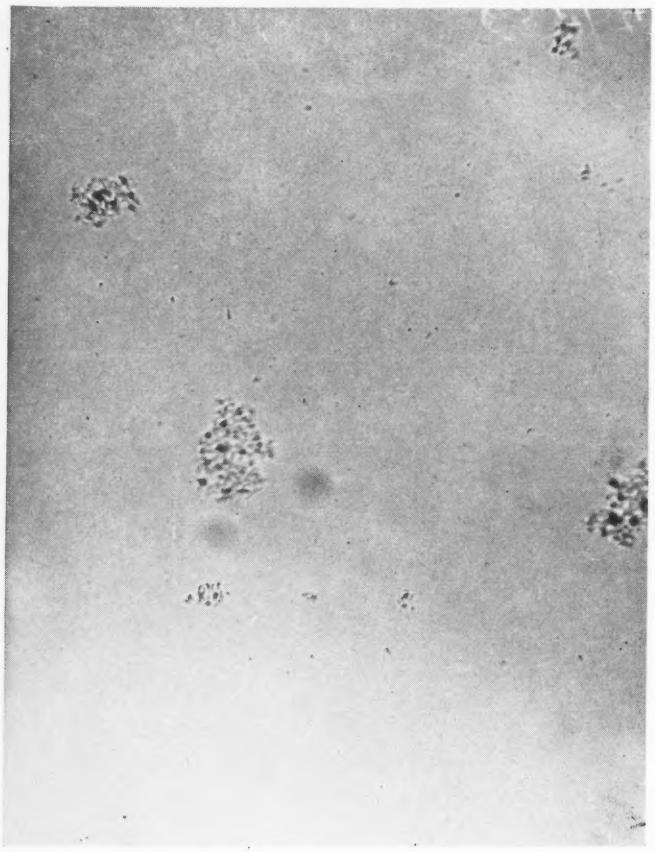

Fig. 3.—Agglutination of platelets by antiserum after six hours. (Magnification ×150.)

Different grades of intensity of agglutination could be observed when the same antiserum was used for agglutination of different samples of platelets. During this study thirteen samples of platelets were found which did not show agglutination by antiserum within three hours. Repeated examinations showed that in four samples of platelets no agglutination could be observed even after six hours. In these last four cases, only by adsorption experiments was it possible to verify the antigenic structure of the platelets.

SUMMARY

The existence of four blood groups of human blood platelets has been established, corresponding to the four major blood groups in human erythrocytes.

Identity of the A and B antigens in platelets and red blood cells has been proved by adsorption of isoagglutinins with erythrocytes and thrombocytes, inhibition of isoagglutinins with saliva, and neutralization of sera with group specific substances.

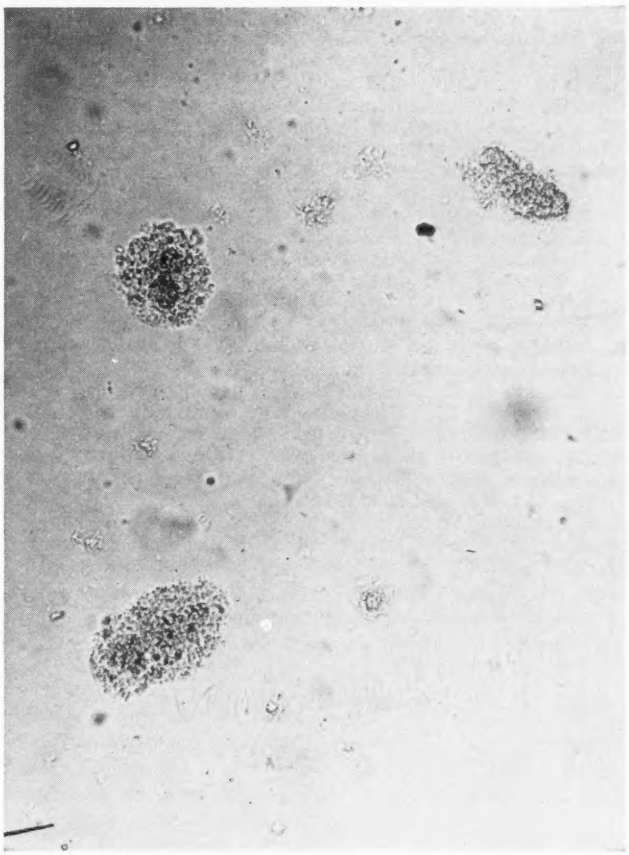

Fig. 4.—Agglutination of platelets by antiserum after nine hours. (Magnification ×150.)

Correlation exists between intenisty of platelet agglutination and titer of antisera.

Agglutination proceeded more rapidly at low temperature.

Storage of platelet suspensions up to seven days in the refrigerator resulted in gradual loss of agglutinability by antisera.

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THE LOCAL ANESTHETIC ACTION OF CERTAIN AMIDES

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IN THE search for local anesthetic compounds that are longer acting and more stable than those available at present, a series of amides of 3-dialkylaminopropylamines of the generic formula R-CONH-R^1-CHR^2-CHR^3-$N(R^4)_2$ were prepared.[1] R, R,[1] and R^4 may be aryl or alkyl, and R^2 and R^3 may be hydrogen or alkyl. The present communication deals with the pharmacologic comparison of twenty-five substances. Two of these, Compound No. 4 (1-benzamido-1-phenyl-3-piperidinopropane) and Compound No. 16 (α-1-benzamido-2-methyl-1-phenyl-3-piperidinopropane) in Table I, were of sufficient interest to warrant extensive examination.

METHODS

All of the compounds were subjected to our standard screening tests for local anesthetic action.[2] Guinea pigs, rabbits, and mice were used. The duration of anesthesia was determined by instillation of the local anesthetic solution into the guinea pig's eye and by intracutaneous injection into the same animal. These tests were usually carried out simultaneously. The degree of irritation was judged by the reaction obtained following intracutaneous injection into the rabbit. Acute toxicity was elicited in mice by intravenous and subcutaneous injections. At least thirty animals were used for each toxicity test and three each for other tests. All compounds were injected as aqueous solutions of their hydrochloride or hydrobromide.

Corneal anesthesia was tested by the method of Beyer.[3] Skin anesthesia following intracutaneous injection into the guinea pig was determined by our own method.[4] The medical medial lethal dose ($LD_{50} \pm$ Standard Error) was computed by the method of Bliss[5] on the basis of the effects of intravenous or subcutaneous injections in white mice.

The criteria for the evaluation of the degree of irritation[2] caused by intracutaneous injection in white rabbits were:

Indistinguishable from surrounding tissue,	negligible	(-)
Trace of hyperemia or ischemia, or a point of elevation,	slight	(+)
Hyperemia, ischemia, or fibrosis,	mild	(++)
Hyperemia or ischemia of more than 1 cm., or fibrosis with extension,	moderate	(+++)
Necrosis, sloughing, induration, or marked edema,	severe	(++++)

The evaluation of worth-while compounds was completed by the sciatic nerve block in the rabbit, described by Schmitz and Loevenhart,[6] and the brachial plexus block in rats, developed in our own laboratory. The brachial plexus block was accomplished by the injection of 1 ml. of a 1 per cent concentration of the local anesthetic solution into rats. A 24 gauge needle attached to a glass Luer syringe was introduced subcutaneously into the medial surface of the foreleg at the second joint. The point of the needle was then carried to within 1 cm. of the chest wall and an injection was made. Motor paralysis of the foreleg followed immediately. The foot on the injected side folded medially and the animal was unable to move the leg or support itself with it. The duration of anesthesia was considered to have reached an end point when the rat could again support itself and walk.

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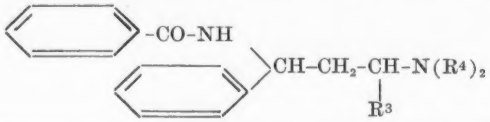
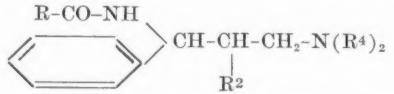
TABLE I. LOCAL ANESTHETIC ACTION OF TWENTY-FIVE AMIDES

Section A



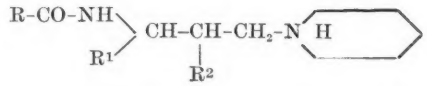
NO.	R^3	$-N(R^4)_2$	DURATION OF ANESTHESIA GUINEA PIGS		IRRITATION RABBIT'S SKIN	MICE LD_{50} ± STANDARD ERROR	
			EYES (MIN.)	SKIN (MIN.)	DEGREE	I. V. (MG./KG.)	S. C. (MG./KG.)
1	H	$-NC_4H_8$ (a)	33	52	++	17 ± 0.5	95 ± 15
2	CH_3	$-NC_4H_8$ (a)	57	75	+	15 ± 0.9	64 ± 3
3	H	$-N(CH_3)_2$	37	91	++	59 ± 3.0	186 ± 26
4	H	$-NC_5H_{10}$ (b)	39	180	-	23 ± 1.0	171 ± 20
5	CH_3	$-NC_5H_{10}$ (b)	66	89	+++	13 ± 0.5	79 ± 13
6	H	$-N(C_4H_9)_2$	60	203	++	9 ± 1.0	154 ± 17

Section B



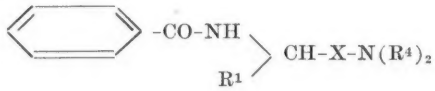
NO.	R	R^2	$N(R^4)_2$					
7	CH_3	H	NC_5H_{10} (b)	0	18	+++	64 ± 1	>1000
8	C_6H_5	CH_3	$N(CH_3)_2$	19	23	++++	57 ± 1	205 ± 27
9	$(CH_3)_2NC_6H_4$	H	$N(CH_3)_2$	32	61	++++	91 ± 5	323 ± 22
10	$4\text{-}NH_2\text{-}C_6H_4$	H	NC_5H_{10} (b)	78	79	++++	14 ± 2	149 ± 14
11	C_6H_5	H	$N_2\text{-}CH_3C_5H_{10}$ (c)	83	156	-	14 ± 1	240 ± 19
12	$C_6H_4OC_6H_4$	H	NC_5H_{10} (b)	515	432	++++	40 ± 2	540 ± 70
13	$C_6H_{11}OC_6H_4$	CH_3	$N(CH_3)_2$	*43	540	++++	54 ± 1	550 ± 13

Section C



NO.		R	R^1	R^2	DURATION OF ANESTHESIA GUINEA PIGS EYES (MIN.)	DURATION OF ANESTHESIA GUINEA PIGS SKIN (MIN.)	IRRITATION RABBIT'S SKIN DEGREE	MICE LD_{50} ± STANDARD ERROR I. V. (MG./KG.)	MICE LD_{50} ± STANDARD ERROR S. C. (MG./KG.)
14	α	C_6H_5	CH_2CH_3	CH_3	0	0	–	45 ± 3.0	190 ± 4
15	β	C_6H_5	CH_2CH_3	CH_3	39	38	–	36 ± 1.0	142 ± 24
16	α	C_6H_5	C_6H_5	H	73	417	–	19 ± 0.2	155 ± 27
17	β	C_6H_5	C_6H_5	H	55	87	–	25 ± 0.4	420 ± 15
18	α	C_6H_5		$`C_4H_8´$ (d)	66	525	++	22 ± 0.1	70 ± 5
19	β	C_6H_5		$`C_4H_8´$ (d)	27	336	++	33 ± 0.8	620 ± 20
20	α	$C_6H_{11}OC_6H_4$	C_6H_5	CH_3	390	390	++++	16 ± 1.0	158 ± 6
21	β	$C_6H_{11}OC_6H_4$	C_6H_5	CH_3	191	430	+++	48 ± 2.0	968 ± 52
22		C_6H_5	$CH_2C_6H_5$	CH_3	56	420	+++	16 ± 1.0	150 ± 11

Section D



NO.	R^1	X	$N(R^4)_2$					
23	C_6H_5	$-(CH_2)_3-$	$N(C_2H_5)_2$	80	380	++	12 ± 1	170 ± 17
24	C_6H_5	$-CH_2-$	NC_5H_{10} (b)	125	476	+++	9 ± 1	90 ± 11
25	$-C_6H_5OCH_3$	$-(CH_2)_2-$	$N(CH_3)_2$	38	79	+++	76 ± 6	152 ± 9

(a) Pyrrolidine.
(b) Piperidine.
(c) 2-Methylpiperidine.
(d) 1-Benzamide-2-piperodinomethyl cyclohexane.
*0.25 per cent.

Intrathecal injections into dogs according to the method of Dvorak and Manson[7] indicated the relative efficiency of these new compounds in spinal anesthesia. Irritating properties were demonstrated by deep injections into the thigh muscles of dogs, and were graded by the impairment of movement and post-mortem findings. Blood pressure, respiratory effects, and intestinal motility after intravenous injections of these preparations were investigated in anesthetized dogs. Electrocardiographic studies were also made on anesthetized cats and dogs.

Prolongation of analgesia was determined by means of a modification of the rat-tail method of Davies[8] and prolongation of hypnosis was measured on the same species. Antipyretic action was looked for in rats made febrile by the subcutaneous injection of a suspension of brewer's yeast. Anti-inflammatory tests were carried out in rats whose feet and ankles had been rendered inflamed by injections of silver nitrate solutions. Eosinopenic action was studied in adrenalectomized mice.

Antispasmodic effects were determined on the isolated guinea pig's ileum.

Acute toxicity data were obtained by intramuscular, intravenous, subcutaneous, and oral dosage of the anesthetics into mice and rats. The mice weighed from 14 to 18 grams and the rats, from 80 to 100 grams.

In subacute toxicity tests, five groups of five female rats each were injected intramuscularly daily for ninety-one days. Ten dogs were similarly injected and observed for ninety-five days.

RESULTS

Representative members of a series of basic amides prepared from 3-dialkylaminopropylamine have been tested. In the series of benzamide derivatives, shown in Table I, Section A, optimal activity accompanies the piperidine and dibutylamino preparations. Substitution of a methyl group alpha to the amino function causes little change in the local anesthetic activity in this group of compounds. Compound No. 4, 1-benzamide-1-phenyl-3-piperidinopropane, is interesting because of the absence of irritating properties when injected into the rabbit's skin.

The results shown in Section B point out the changes in local anesthetic activity produced by the modification of the amide portion of the molecule. The acetamide derivative, Compound No. 7, is much less active than the benzamide or the substituted benzamides. Cyclohexyloxy substitution of the benzamide sometimes enhances local anesthetic action.

Section C illustrates the effect of alkyl substitution beta to the amino function. The diastereo-isomeric pairs of four compounds are designated as α and β. The α-diastereomer of 1-benzamido-2-methyl-1-phenyl-3-piperidinopropane, Compound No. 16, is significantly more active than the β-isomer. This difference is not nearly so pronounced in the corresponding cyclohexyloxy benzamides, Compounds No. 20 and No. 21. Where R^1 and R^2 form a cyclohexane structure, the anesthetic potency in guinea pig's skin is still of the same magnitude. Only one diastereo-isomer of Compound No. 22, in which R^2 is benzyl instead of phenyl, was prepared. An unusual feature of this series is the lack of irritating properties in the first two pairs.

Test results of three compounds in which the chief changes are found in the aliphatic mid-chain are entered in Section D. The phenylethane derivative is the most active, the *p*-methoxyphenylpropane the least, and the toxicity is proportional to the activity.

Two of the twenty-five compounds deemed especially worthy of further study were Compound No. 4 in Section A, 1-benzamido-1-phenyl-3-piperidinopropane hydrochloride, and Compound No. 16 in Section C, α-1-benzamido-2-methyl-1-phenyl-3-piperidinopropane hydrochloride. Compound No. 4 is capable of producing profound anesthesia when applied topically to mucous membranes, when injected parenterally or intrathecally, or when used as an immersion medium for an exposed nerve.

Duration of Anesthesia.—The direct anesthetic action of Compound No. 4 is summarized in Table II. It will be noted that adequate duration of anesthesia results from: intradermal injection of a 0.5 per cent solution into guinea pig's skin; the application of a 1 per cent solution to guinea pig's eyes; the injection of a 1 per cent solution into the area of the rat's brachial plexus; the application of a 0.15 per cent solution on the rabbit's exposed sciatic nerve; the intrathecal injection of a 1 per cent solution into the dog; and the injection of a 2 per cent solution into the rabbit's lower lip.

Irritation.—Studies on the irritating properties of Compound No. 4 were done by intramuscular injection into the thighs of dogs. Six tests in all were made, and the resulting irritation was negligible.

Stability.—The results of a six-week stability test on a solution of this compound are found in Table III. There is no significant difference between the least and greatest toxicity figures, nor among the results for duration of anesthesia.

Acute Toxicity.—Acute toxicity experiments, Table IV, were carried out on mice and rats. There is little species difference except in the case of oral toxicity in the mouse, which in turn differs little from the intramuscular toxicity value in that animal. We believe that this indicates a very rapid rate of absorption of Compound No. 4 from the gut, particularly in the mouse. The intravenous toxicity figures for the mouse are reported in Section A of Table I, and in Table III.

Antagonism.—The acute toxicity of local anesthetic compounds is usually decreased in the presence of barbiturates. Table V shows the effects of concurrent subcutaneous injection of Compound No. 4 and Sodium Amytal* into 30 gram mice.

Sodium Amytal definitely protects mice against the acute toxic action of Compound No. 4. Although there is no apparent difference between the action of 80 and 120 mg. per kilogram doses of the barbiturate, there is a significant difference between the LD_{50}'s of these dose combinations and the control group receiving only the local anesthetic.

Subacute Toxicity, Rats.—Subacute toxicity experiments by intramuscular injection into female rats in which four groups of five animals each received varying amounts of Compound No. 4, and the fifth group of five received saline, showed the following:

The weight gain was increased slightly in the rats injected with 10 mg. per kilogram daily, and slightly retarded in the animals on 20 and 40 mg. doses per

*Amobarbital sodium, Eli Lilly & Company.

TABLE II. MEAN DURATION OF ANESTHESIA, COMPOUND NO. 4

METHOD	NO. OF ANIMALS	PER CENT SOLUTION	DOSE (ML.)	ANES-THESIA (MIN.)	PER CENT SOLUTION	ANES-THESIA (MIN.)	PER CENT SOLUTION	ANES-THESIA (MIN.)	PER CENT SOLUTION	ANES-THESIA (MIN.)
Topical Anesthesia (Applied to guinea pig's eyes)	30	1	0.1	39.4	0.5	17.1	0.25	None		
Infiltration Anesthesia (Intradermal injection in guinea pigs)	40	1	0.1	180.5	0.5	91.4	0.25	27.9	0.1	7
Motor Nerve Block (Brachial plexus block in the rat)	10	1	1.0	109.0						
Sensory Nerve Block (Sciatic nerve block in the rabbit)	10	0.15	2.0	30.9						
Intrathecal Anesthesia (Spinal block in the dog)	7	1	0.15/kg.	55.0						
Depot Anesthesia (S. C. injection in the lower lip of rabbits)	3	2	0.5	8 (days)						

TABLE III. STABILITY OF COMPOUND NO. 4

TIME	ROOM TEMPERA-TURE	LD_{50} ± S. E. MICE I. V. (MG./KG.)	NO. OF ANIMALS	DURATION OF ANESTHESIA IN GUINEA PIGS							
				PER CENT SOLUTION	EYES (MIN.)	PER CENT SOLUTION	EYES (MIN.)	PER CENT SOLUTION	SKIN (MIN.)	PER CENT SOLUTION	SKIN (MIN.)
1st wk.	26.1° C.	17.5 ± 0.7	6	1	37	0.5	18	0.5	82	0.25	36
2nd wk.	26.1° C.	17.3 ± 0.8	3	1	40	0.5	20	0.5	90	0.25	41
3rd wk.	26.1° C.	16.4 ± 0.6	3	1	32	0.5	17	0.5	96	0.25	41
4th wk.	26.7° C.	16.8 ± 0.8	3	1	44	0.5	21	0.5	90	0.25	39
5th wk.	26.7° C.	20.2 ± 1.1	3	1	42	0.5	21	0.5	94	0.25	45
6th wk.	26.1° C.	19.7 ± 1.3	3	1	38	0.5	21	0.5	88	0.25	37

kilogram as compared with the saline controls. All of the rats on 80 mg. per kilogram doses died after 2, 5, 8, 8, and 11 days, respectively. Necropsy failed to reveal the cause of death. Two animals had some pulmonary edema and others showed mild injury to the skeletal muscles at the site of injection.

All survivors were sacrificed and submitted to necropsy after ninety-one days of treatment. Gross and microscopic studies of the viscera showed no abnormalities with the exception of some bronchopneumonia in one saline-treated control animal. Rats that received 20 or 40 mg. per kilogram daily had some slight intramuscular fibrosis with atrophy of a few muscle fibers. The daily dose volume per rat in these groups ranged from 0.075 to 0.15 ml. of a 2.5 per cent aqueous solution containing 0.5 per cent chlorbutanol.

TABLE IV. ACUTE TOXICITY OF COMPOUND NO. 4 IN MICE AND RATS

SPECIES	METHOD OF ADMINISTRATION	LD_{50} ± S. E. (MG./KG.)
Mouse	Intramuscular	95.6 ± 2.8
	Oral	98.5 ± 7.8
Rat	Intravenous	16.2 ± 0.79
	Subcutaneous	111.6 ± 16.3
	Intramuscular	119.8 ± 11.6
	Oral	222.1 ± 28.9

TABLE V. ACUTE TOXICITY OF COMPOUND NO. 4 IN MICE WITH AND WITHOUT SODIUM AMYTAL

SUBCUTANEOUS DOSES OF COMPOUND NO. 4 (MG./KG.)	SUBCUTANEOUS DOSES OF SODIUM AMYTAL (MG./KG.)	LD_{50} ± S. E. MG./KG.
225, 200, 175, 145	None	181.5 ± 4.9
455, 345, 265, 200	80	269.8 ± 15.1
400, 300, 250, 200	120	254.5 ± 13.0

Note: These tests were performed on adult mice, which may account for the elevated toxicity level of the control group of mice.

Subacute Toxicity, Dogs.—Preliminary studies indicated that daily intramuscular doses of 5 and 10 mg. per kilogram of Compound No. 4 were well tolerated by dogs. Ten dogs were used. All but one dog in the higher dose group showed some weight gain over the period of treatment. This one animal had mild convulsions within ten minutes after an intramuscular dose of 10 mg. per kilogram. Blood and urine samples were taken at two-week intervals for various analyses. The erythrocyte, leukocyte, and differential counts, as well as the hemoglobin and hematocrit values, nonprotein nitrogen, blood and urine sugar, urine albumin, and whole blood-clotting time were determined. Normal fluctuations were apparent in all the studies, but no significant change was demonstrated in any case.

All dogs survived the ninety-five-day treatment period and were sacrificed and submitted for necropsy. Gross and microscopic examination showed all visceral tissues to be normal. Although the same solution was used here as that reported for the rats, the muscles at the sites of injection were uninjured.

TABLE VI. DURATION OF ANESTHESIA OF COMPOUND NO. 16

METHOD	NO. OF ANIMALS	DOSE (ML.)	PER CENT SOLUTION	ANESTHESIA (MIN.)	PER CENT SOLUTION	ANESTHESIA (MIN.)	PER CENT SOLUTION	ANESTHESIA (MIN.)	PER CENT SOLUTION	ANESTHESIA (MIN.)	PER CENT SOLUTION	ANESTHESIA (MIN.)
Topical anesthesia	18	0.1	2	183	1	73	0.5	58	0.25	29	0.1	12	0.05	0
Infiltration anesthesia	18	0.1	2	540	1	417	0.5	346	0.25	245	0.1	75	0.05	49
Sensory nerve block	3	2.0	0.05	45										
Intrathecal anesthesia	3	0.15 /kg.	1.0	71										

Electrocardiographic Studies.—Five milligrams of Compound No. 4 per kilogram, by slow intravenous infusion into anesthetized dogs, caused an increase in heart rate with inversion of the T wave. A dose of 10 mg. per kilogram produced an increase in heart rate with changes in both the T and P waves. In one dog this dose caused auriculoventricular dissociation.

Arrhythmia.—In cats anesthetized with chloroform and carried at Stage III, Plane 2 anesthesia, Compound No. 4 by intravenous injection of 1 to 3 mg. per kilogram, gave transient protection against epinephrine-chloroform induced arrhythmias. Following the short period of protection, however, the animals appeared to be more sensitive to this type of induced cardiac irregularity.

Analgesia.—Solutions of Compound No. 4 in subcutaneous doses of 10 to 80 mg. per kilogram, intraperitoneal doses of 5 to 40 mg. per kilogram and intravenous doses of 2 to 20 mg. per kilogram produced only a trace of analgesia in the rat. The higher doses were sometimes fatal. Subcutaneous doses of 25 mg. per kilogram prolonged the action of Dolophine Hydrochloride* from two to four hours in the same species.

Miscellaneous Effects.—Blood pressure, respiration, and intestinal motility were determined on dogs anesthetized with Seconal Sodium† or with phenobarbital, and then injected with Compound No. 4. Intravenous doses of 5 to 10 mg. per kilogram caused a temporary fall in blood pressure, a slight but definite slowing of respiration after an initial increase in the rate and volume, and a reduction in intestinal motility. The degree of the reactions depended largely upon the concentration of the drug and the rate of injection. A dose of 10 mg. per kilogram in a 1 per cent solution, injected at the rate of 1 ml. per minute, caused a very slight fall in blood pressure, but 6 mg. per kilogram in a 2.5 per cent solution, injected at the same rate, brought about a marked depression of blood pressure and respiration. The action on intestinal motility was at no time complete, regardless of the effect on blood pressure or respiration.

Compound No. 4 had neither antipyretic nor anti-inflammatory action in rats. There was no eosinopenic action following subcutaneous doses of 50 and 100 mg. per kilogram in adrenalectomized gray mice (Indiana University Strain); no prolongation of hypnosis occurred when the drug was given in subcutaneous doses of 25 mg. per kilogram, plus intravenous doses of 100 mg. of Evipal per kilogram of body weight; and no measurable antispasmodic action when tested on the isolated guinea pig ileum.

A summary of the more important pharmacologic actions of Compound No. 16 (Section C in Table I) is to be found in Table VI.

Duration of Anesthesia.—Adequate local anesthesia was obtained with a concentration of 0.25 per cent on guinea pig eyes; and with a 0.05 per cent concentration in guinea pig skin. A 0.05 per cent solution definitely anesthetized the rabbit's sciatic nerve for forty-five minutes; and spinal anesthesia of more than one hour followed the use of a 1 per cent solution in dogs.

*Methadon hydrochloride, Eli Lilly & Company.
†Secobarbital sodium, Eli Lilly & Company.

Analgesia.—Subcutaneous doses of 10 to 20 mg. per kilogram produced a mild and inconsistent analgesia in rats. Doses of 40 mg. per kilogram caused ataxia and 80 mg. per kilogram, death. Twenty-five mg. per kilogram, given by subcutaneous injection, prolonged the action of Dolophine Hydrochloride slightly more than 50 per cent in rats.

Miscellaneous Effects.—Oral doses of 25 mg. per kilogram plus subcutaneous doses of 100 mg. per kilogram of Evipal increased the sleeping time of mice by 11 per cent as compared with controls treated with Evipal alone.

DISCUSSION

Twenty-five amides were divided into four classes in which different parts of the molecule were modified. Thus, where the chief changes occur in the amino function, the optimal activity accompanies the piperidine and butylamino compounds.

Cyclohexyloxy substitution of the benzamide markedly increases the local anesthetic action in those compounds in which changes are made in both the amino and benzamide portions of the molecule.

Alkyl substitution beta to the amino function in four pairs of diastereoisomers brings the unexpected feature of lessened irritation in two of the pairs.

Where changes are within the aliphatic mid-chain, the phenylethane derivative appears to be the most active.

SUMMARY

Twenty-five amides have been studied for their local anesthetic properties and other pharmacologic effects. Two of them are particularly active in animals with a minimum of undesirable reactions. They are, chemically: 1-benzamido-1-phenyl-3-piperidinopropane hydrochloride (Compound No. 4) and α-1-benzamido-2-methyl-1-phenyl-3-piperidinopropane hydrochloride (Compound No. 16).

1. Compound No. 4 is stable and active when applied topically, by intradermal and intrathecal injection, and by subcutaneous injection into the region of the brachial plexus.

2. Compound No. 4 is relatively nonirritating and only moderately toxic.

3. Compound No. 16 is a relatively long-acting local anesthetic by topical application and by intradermal or intrathecal injection.

4. Compound No. 16 is comparatively nonirritating and its toxicity is of the same order as that of Compound No. 4.

We wish to express our thanks to Dr. R. C. Anderson for the acute and chronic toxicity studies; to Dr. F. G. Henderson for the electrocardiographic work; and to Dr. P. N. Harris for the gross and microscopic examination of necropsy material.

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STUDIES OF THE ABSORPTION, DISTRIBUTION, AND EXCRETION OF ACETYL SULFISOXAZOLE, AN INSOLUBLE SULFONAMIDE APPEARING IN SULFISOXAZOLE IN THE BLOOD

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THE introduction of the more soluble forms of the sulfonamides reduced significantly the incidence of renal complications associated with their administration.[1] This greater solubility is accompanied by an increase in the bitter taste of the liquid preparations, however, thereby making the administration to children more difficult. In an attempt to solve this problem, an insoluble acetyl derivative of sulfisoxazole was prepared. Following the oral administration of a suspension of this compound, sulfisoxazole appears in the blood. The conversion presumably takes place in the intestinal tract.

METHODS OF STUDY

Acetyl sulfisoxazole, an N^1 acetyl derivative of sulfisoxazole, was supplied as a vanilla-flavored suspension containing 593 mg. per 5 ml., and as tablets containing 590 mg. This concentration was calculated as the molecular equivalent of 500 mg. of sulfisoxazole. Only the aqueous suspension was given to children.

The following study was undertaken to determine: (1) the palatability and patient acceptance of acetyl sulfisoxazole; (2) the time of peak blood levels following a single dose; (3) duration of blood levels; (4) twenty-four-hour urinary excretion; and (5) the levels in other body fluids as compared to blood levels. Twenty-nine children moderately ill or convalescing from acute infections were selected from hospitalized patients for this study.

After drawing a control blood specimen, single oral doses of acetyl sulfisoxazole suspension were given to thirteen children ranging from 6 months to 13 years of age. Blood specimens were drawn at one-, three-, six-, twelve-, and twenty-four-hour intervals. The drug dosage used ranged from 36 to 100 mg. per kilogram. In two children equal doses of sulfisoxazole and acetyl sulfisoxazole were given and the blood levels compared.

Sixteen children were placed on a multiple-dose schedule. The age of these children was from 13 days to 8 years. A stomach tube was used to administer the suspension in infants. The total twenty-four-hour dose ranged from 135 to 400 mg. per kilogram and was divided into four equal doses given at six-hour intervals. The drug was given in consecutive doses for two to ten days and daily samples were obtained to determine blood levels. The blood was drawn from all patients receiving multiple doses just before the next dose was administered.

Eighteen adult bed patients convalescing from a variety of illnesses were given single oral doses of acetyl sulfisoxazole in tablet form ranging from 0.5 to 4 Gm. Blood levels were measured at thirty minutes, one, three, six, twelve, and twenty-four hours, and the urinary excretion was determined in fourteen of these patients during the first twenty-four hours following the administration of the drug.

Seven adults were placed on a multiple-dose schedule with the dose ranging from 1 to 3 Gm. every four hours. Daily blood samples were drawn within an hour before the first

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morning dose. In five patients, both free and acetylated forms of the drug in the blood were determined. Two patients received the suspension of acetyl sulfisoxazole and five received the tablet form. Twenty-four-hour urine samples were collected daily and urinary excretion of the drug determined. In one adult, simultaneous blood and knee-joint fluid levels were obtained. In a second adult, who did not have meningitis, simultaneous spinal fluid and blood levels were measured. In a third patient, simultaneous pleural fluid and blood levels were obtained on two occasions.

In the adult patients venous blood was collected in oxalated tubes and stored at 5° C. for two to three days or frozen if held for longer periods. Urine specimens were stored at 5° C. In the children blood was obtained either by drawing venous samples or by collecting capillary blood by means of the heel puncture technique.

The Bratton and Marshall method of determining sulfonamide levels was followed, using the photoelectric colorimeter method outlined in Hepler's *Manual of Clinical Laboratory Methods.*[2] Sulfisoxazole was used in setting up the standard curve. In infants, capillary blood was analyzed by the micromethod described in Bugy's *Synopsis of Clinical Laboratory Methods.*[3]

RESULTS

The blood levels in thirteen children receiving a single dose of acetyl sulfisoxazole suspension are shown in Table I. Significant blood levels were obtained when the dose administered was 72 mg. per kilogram or greater. In Fig. 1 are presented the average blood levels of five children receiving 100 mg. per kilogram as a single dose. The maximal blood concentration with acetyl sulfisoxazole was attained between the third and the sixth hour following administration of the drug.

TABLE I. BLOOD LEVELS OF FREE SULFONAMIDE IN THIRTEEN CHILDREN RECEIVING A SINGLE DOSE OF ACETYL SULFISOXAZOLE SUSPENSION

PATIENT	DOSE (MG. / KG.)	WEIGHT (KG.)	BLOOD LEVEL (MG. PER CENT)					
			1 HR.	3 HR.	5 HR.	6 HR.	12 HR.	24 HR.
F. R.	36	8.9	1.0	4.9		3.3		0.5
D. C.	37	7.9	3.0	5.4	4.4		2.2	0.6
B. S.	53	38.0	0.4	1.2		0.7		0.2
F. R.	72	8.9	4.9	9.1		7.0		0.3
D. C.	77	7.9	5.0	7.6		6.7		1.4
P. T.	78	9.0	3.9	13.7		16.3		2.6
A. H.	83	12.0	3.0	10.6	11.0	7.3		0.4
S. M.	89	9.8	3.0	6.4		12.3		2.0
C. B.	100	5.0	2.9	5.9		2.9	2.8	0.5
B. D.	100	10.0	5.8	11.5		9.4	5.2	2.0
L. A.	100	10.0	3.6	9.3		6.4	5.2	1.3
L. A.	100*	10.0	2.5	8.3	13.1	15.6	7.6	1.4
H. Y.	100	23.0	2.3	12.4		10.1	7.1	2.1
H. Y.	100*	23.0	1.1	3.8	18.4	18.4	10.2	1.8
R. G.	100	30.0	1.8	6.8		2.7	3.5	1.4

*Unacetylated sulfisoxazole administered.

The twelve-hour level was 4.8 mg. per cent (Fig. 1) as compared to a level of 2 mg. per cent obtained with sulfisoxazole by Price and Hansen.[4] The mean peak level of 9.2 mg. per cent was reached at three hours, this concentration being similar to the average maximal level at three hours obtained by Price and Hansen[4] in their sulfisoxazole studies.

TABLE II. BLOOD LEVELS OF FREE SULFONAMIDE IN SIXTEEN CHILDREN RECEIVING ACETYL SULFISOXAZOLE SUSPENSION EVERY SIX HOURS

PATIENT	WEIGHT (KG.)	DOSE (MG./KG. DAY)	BLOOD LEVEL (MG. PER CENT)											
			1		2		3		4		5		6		7 DAYS	
			A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.
R. T.	20.5	135	6.7		8.8		10.4									
D. C.	7.9	150	4.4		5.8		6.0									
L. W.	13.2	180	4.6		10.4		9.0									
P. M.	4.4	200		2.9	2.9		6.9		3.2							
V. S.	10.2	240	1.9	2.4	6.4	5.8	8.0									
V. C.	3.0	300	7.0		7.4		5.3									
M. H.	7.3	300	2.6	6.0	7.4	3.2	4.8		3.4		4.6					
V. G.	5.0	320	8.6	9.0	8.3		16.0		13.1		6.5					
R. C.	10.6	360	2.9	4.2	2.6		3.7	6.4	4.7		25.4		17.2			
W. S.	23.2	400	0.6	5.0	20.5	23.2	16.0		19.2	16.0	12.0		9.3		3.4	
M. C.	10.0	400	14.8		10.8		4.1		8.3		15.6		8.1			
T. C.	22.7	400	26.4		24.6		21.2									
J. B.	10.0	400	0.3		2.1		5.9		5.4							
F. R.	9.0	400		7.5	1.7		1.1		12.0							
J. M.	24.0	400		18.6	17.2		14.0		11.8							
D. L.	10.3	400	4.6	12.0												

The blood levels obtained in sixteen children receiving 135 to 400 mg. of acetyl sulfisoxazole suspension per kilogram per day in equally divided dosage at six-hour intervals are presented in Table II. In those children who received 135 to 240 mg. per kilogram per day the blood levels ranged from 1.9 to 10.4 mg. per cent with an average of 5.9 mg. per cent. When the dose was increased to 300 to 400 mg. per kilogram per day, the blood levels varied from 0.3 to 26.4 mg. per cent, with an average of 9.4 mg. per cent. When a comparison is made with the data reported by Price and Hansen,[4] who administrated 200 mg. of unacetylated sufisoxazole per kilogram per day under somewhat different conditions, it appears that a larger dose of acetyl sulfisoxazole is required to obtain equivalent daily peak blood levels.

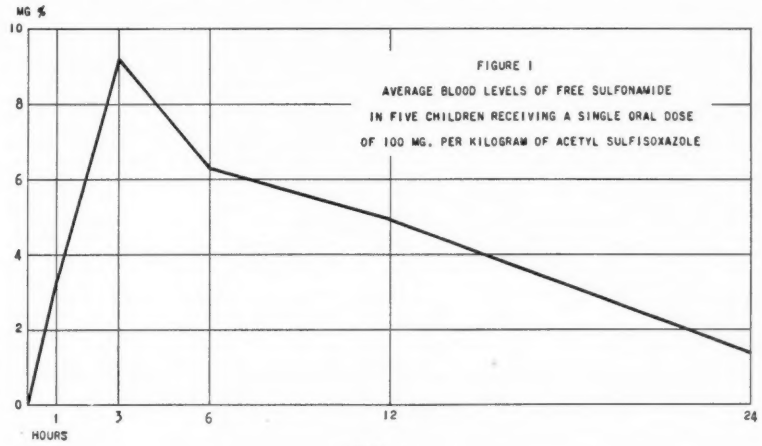

Fig. 1.

The levels of free sulfonamide obtained in adults following single doses of 0.5 to 4.0 Gm. of acetyl sulfisoxazole are presented in Table III. As can be seen, relatively low maximum blood levels resulted from the administration of the drug in these doses. Sarnoff[5] obtained peak levels of 14 to 16 mg. per cent after single oral doses of 3.0 and 4.0 Gm. of sulfisoxazole. Svec, Rhoads, and Rohr[6] obtained blood levels of 9 mg. per cent using a single oral dose of 2.0 Gm. of sulfisoxazole. Brickhouse and associates,[7] on the other hand, reported an average maximum level of 10.7 mg. per cent using a dose of 6 Gm. In this series of adults given 4.0 Gm. of acetyl sulfisoxazole in a single dose, the average peak level obtained was 4.4 mg. per cent. This suggests that in adults acetyl sulfisoxazole is absorbed less rapidly than sulfisoxazole. The urinary excretion of the drug in the first twenty-four hours averaged 35 per cent of the single dose in this study.

The blood levels of sulfisoxazole in three adults given 1 Gm. and four adults given 3 Gm. every four hours are illustrated in Table IV. The average level obtained with 1 Gm. every four hours was 5.4 mg. per cent and with 3 Gm. every four hours, 8.7 mg. per cent. In both adults and children, there was a marked variation of blood levels of different patients receiving similar doses and a gradually increasing level was obtained upon continuous administration.

TABLE III. BLOOD LEVELS AND URINARY EXCRETION OF FREE SULFONAMIDE IN EIGHTEEN ADULTS RECEIVING SINGLE DOSE OF ACETYL SULFISOXAZOLE TABLETS

PATIENT	DOSE (GM.)	BLOOD LEVEL (MG. PER CENT) ½ HR.	1 HR.	3 HR.	6 HR.	12 HR.	24 HR.	AMOUNT EXCRETED IN URINE FIRST 24 HR. (PER CENT)
P. E.	0.5	0.0	0.1	0.4	1.6	1.6	0.4	44
R. E.	0.5	0.3	0.7	2.8	1.7	0.8	0.6	12
F. L.	1.0	0.4	0.6	1.9	2.7	2.0	1.2	
B. C.	1.0	0.8	1.0	2.0	2.6	1.2	0.7	
H. A.	1.5	0.4	1.7	2.8	2.3	1.3	0.5	60
C. A.	1.5	0.6	0.7	1.7	4.2	3.3	1.7	15
D. A.	2.0	0.3	0.3	2.7	3.6	1.9	0.9	100
D. U.	2.0	0.2	0.6	1.4	2.1	1.7	0.9	10
H. A.	2.5	0.5	0.5	0.8	1.1	1.7	1.3	7
D. E.	3.0	0.9	3.0	6.6	4.6	1.8	0.6	
H. A.	3.0	0.9	2.3	5.2	4.5	2.7	1.3	49
S. M.	3.0	0.3	0.2	1.1	2.1	2.5	1.0	2
B. E.	3.0	0.1	0.3	4.8	4.4	2.6		
F. I.	3.5	0.5		5.3	4.5	3.2	3.2	2
C. H.	4.0	0.0	0.1	2.7	0.7	4.1	2.2	28
M. R.	4.0	0.0	0.7	5.3	0.5	2.4	0.6	64
A. D.	4.0	0.4	0.9	4.5	5.3	2.4	0.9	90
R. B.	4.0	0.1	0.1	0.1	0.3	1.3	2.9	10

TABLE IV. DAILY BLOOD LEVELS OF FREE SULFONAMIDE IN SEVEN ADULTS RECEIVING MAINTENANCE DOSAGE OF ACETYL SULFISOXAZOLE

PATIENT	DOSE (GM. EVERY HOUR)	BLOOD LEVEL (MG. PER CENT) 1	2	3	4	5	6	7	8	9	10 DAYS
B. C.	1	2.4	4.5	6.4	5.4	5.3	3.1	5.1	4.1	5.4	7.2
S. E.	1	1.2	5.1	5.3	6.7	4.2					
E. D.	1	6.5	6.3	8.1	7.3						
R. B.	3	4.7	7.6	7.4	6.1	4.9	11.8	11.4	4.7	13.4	7.2
W. H.	3	4.9	4.9	5.7	1.6	5.1					
R. O.	3	13.4	8.6	6.5	3.7	6.6	13.0	18.2	18.0	15.2	16.2
G. R.	3	11.9	8.3	11.9	9.9	14.3	15.5	15.1	10.3	3.4	3.7

The determination of the free and total blood sulfonamide in five patients receiving acetyl sulfisoxazole indicated that 15 per cent was acetylated (Table V), whereas Rhoads and his co-workers[8] in a study of ten patients who ingested sulfisoxazole found the average percentage of acetylated sulfonamide in the blood to be 35 per cent.

TABLE V. TOTAL AND FREE SULFONAMIDE BLOOD LEVELS IN FIVE ADULTS ON DAILY DOSAGE OF ACETYL SULFISOXAZOLE TABLETS

PATIENT	DAILY DOSE (GM. EVERY 4 HR.)	SULFONAMIDE BLOOD LEVELS (MG. PER CENT) FREE	TOTAL	PER CENT CONJUGATED
B. E.	1	6.3	7.9	20
R. B.	3	4.7	4.9	4
R. W.	3	4.9	5.3	9
	3	4.9	6.1	19
F. R.	3	13.4	16.2	17
	3	8.6	12.0	28
M. G.	3	11.9	13.4	11
Average		7.8	9.4	15

A comparison of the sulfonamide blood levels and levels in various body fluids in three patients is presented in Table VI. The average concentration in the pleural fluid was 63 per cent that of the blood level in one patient. The spinal fluid level was found to be 57 per cent of the blood level in another patient, and the knee joint fluid level was found to be 65 per cent that of the blood level in a third patient.

TABLE VI. SIMULTANEOUS BLOOD AND BODY FLUID LEVELS IN THREE ADULTS RECEIVING DAILY DOSAGE OF ACETYL SULFISOXAZOLE TABLETS

PATIENT	DAILY DOSE (GM. EVERY 4 HR.)	DAYS THERAPY	BLOOD LEVEL (MG. PER CENT)	BODY FLUID LEVEL (MG. PER CENT)	BODY FLUID
R. B.	3	4	11.8	1.8	Pleural
	3	6	4.7	9.1	Pleural
M. G.	3	3	11.9	6.8	Cerebrospinal
B. C.	1	6	7.2	4.7	Knee-joint

Frequent determination of total and differential leukocyte counts, hemoglobin, and erythrocyte counts on peripheral blood showed no deleterious effect of acetyl sulfisoxazole in any case. No rashes or other evidences of idiosyncrasy to the drug were noted. Frequent urinalyses on all patients receiving the drug showed no evidence of hematuria, crystalluria, or albuminuria. No child refused acetyl sulfisoxazole suspension because of unpleasant taste and the drug was consumed with relish in most cases. The large number of tablets required to maintain high blood levels was objectionable to most adults.

CONCLUSIONS

Acetyl sulfisoxazole, an N^1 acetyl sulfisoxazole, which is apparently converted to sulfisoxazole in the gastrointestinal tract, was given to twenty-nine children in single and multiple doses. Blood levels were obtained with an average peak level at three hours of 9.2 mg. per cent after single doses of 100 mg. per kilogram. Higher blood levels are encountered at twelve and twenty-four hours after administration of a single dose, suggesting that acetyl sulfisoxazole is absorbed more slowly than sulfisoxazole. Larger amounts of acetyl sulfisoxazole must be given to obtain maximum levels comparable to those attained after unacetylated sulfisoxazole is administered. Average blood levels of 9.4 mg. per cent were reached in children on maintenance doses of 300 to 400 mg. per kilogram per day.

The studies of acetyl sulfisoxazole in adults revealed that satisfactory peak blood levels were not attained by single doses of 4 Gm., but continuous administration of 3 Gm. every four hours produced an average blood level of 8.7 mg. per cent. The drug was excreted relatively slowly, with 35 per cent of a single dose appearing in the urine during the first twenty-four hours. The percentage of the sulfonamide in the blood as the acetylated form was found to average 15 per cent. Satisfactory crossing of body membranes was reflected by the level in knee-joint, pleural, and spinal fluids. No adverse side reactions to the drug were noted and children found the suspension form of the drug to be quite palatable. The large number of tablets required to

maintain maximal blood levels in adults, however, might limit its usefulness in patients with systemic infections in this age group. The final interpretation of the significance of the blood levels in both children and adults will have to be made on the basis of the results of critical clinical evaluation.

SUMMARY

1. An insoluble, tasteless, sulfonamide compound, N^1 acetyl sulfisoxazole, was administered orally to twenty-nine children and twenty-five adults in single and multiple dosage schedules. This appeared as a soluble sulfonamide, sulfisoxazole, in the body fluids.

2. The blood levels were somewhat higher in children than in adults; adequate levels in children being maintained by doses of 300 to 400 mg. per kilogram per day. In adults, daily doses of 3.0 Gm. every four hours produced average blood levels of 8.7 mg. per cent.

3. The levels of the drug in the spinal, pleural, and joint fluid averaged 62 per cent of the blood level.

4. The palatability of the oral suspension was quite acceptable and no undesirable side effects associated with its administration were noted. The usefulness of acetyl sulfisoxazole in the treatment of systemic infections in adults may be limited by the large number of tablets which are apparently necessary.

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ROUTINE TESTING OF THE BACTERICIDAL ACTION OF ANTIBIOTICS FOR CLINICAL PURPOSES

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EXPERIENCE has shown that the minimum concentrations of an antibiotic required to inhibit the growth of different bacterial strains may differ widely from one strain to another, even of the same species. A similar variation in lethal activity also exists, but has not been studied so systematically. The bacteriostatic action of a drug is probably sufficient aid to the body defenses in most types of infection, but it is believed that in some instances, for example, subacute bacterial endocarditis, actual sterilization of the lesions is essential for a permanent cure.[1-3] If this is so, the bactericidal action of antibiotics on routinely isolated organisms should be determined as an aid in the choice of a drug suitable for therapy. Hitherto information on the bactericidal action of drugs has been obtained only by means of viable counting or turbidimetric techniques, which, because of the repeated sampling involved and their laborious nature, have been little used for the investigation of individual problems of this kind. A more convenient method may prove to be useful and should be evaluated in the light of clinical experience.

This paper describes a simple technique which is an extension of the blotting paper disc method for "sensitivity testing."[4] A description of the principles on which it is based, and its application to problems of interaction between antibiotics, has already been made.[5, 6] The method is an adaptation of the technique of "replica plating" described by Lederberg and Lederberg,[7] and used by them for studying problems of bacterial genetics. It consists essentially of the transfer of bacteria, without disturbing their spatial relationships, from one nutrient agar surface to another. The transferring agent is the "pile" of a piece of sterile velvet covering a flat surface: the pile is pressed against the surface carrying the organisms (the "primary" plate) and a proportion of them adhere to the velvet. The infected fabric is then "printed" onto the surface of a sterile agar plate, the "replica" plate. This is incubated in the usual way and the amount and distribution of bacterial growth on it are compared with those on the primary plate.

MATERIALS AND METHOD

Organisms Tested.—Sixty-five strains were tested, consisting of twenty-seven strains of coagulase-positive staphylococci from a variety of human lesions, eighteen strains of *Escherichia coli* from urinary infections, ten strains of enterococci from cases of subacute bacterial endocarditis, and ten strains of hemolytic streptococci (Lancefield groups A, B, and C).

Antibiotics.—Seven antibiotics at present in common use were tested. The blotting paper discs were soaked in concentrations of the drugs as follows: penicillin G, 500 units per milliliter, except when tested against the enterococci, when a concentration of 50,000

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units per milliliter was used; streptomycin, 1,000 μg per milliliter; chloramphenicol, 5,000 μg per milliliter; Aureomycin, 2,500 μg per milliliter; Terramycin, 250 μg per milliliter; bacitracin, 1,000 units per milliliter; and polymyxin B, 10,000 units per milliliter. These concentrations are somewhat arbitrary: other concentrations might be suitable for different media in routine use, or when testing different bacterial species. The aim, when choosing a suitable concentration of antibiotic for the test, is to select that amount which will produce an inhibition zone of 15 to 25 mm. diameter on strains of normal sensitivity. Experience with the technique has shown that erroneous impressions of the bactericidal action of a drug may arise if the size of the inhibition zone does not fall within these limits.

Medium.—Peptone (Evans) 1 per cent, Lab-Lemco 0.5 per cent, NaCl 0.5 per cent, and New Zealand agar (Davis) 2 per cent were dissolved in glass-distilled water and the pH was adjusted to 7.4 to 7.6. The medium was sterilized by autoclaving at 120° C. for fifteen minutes. For cultivation of streptococci, 5 per cent of oxalated horse blood was added.

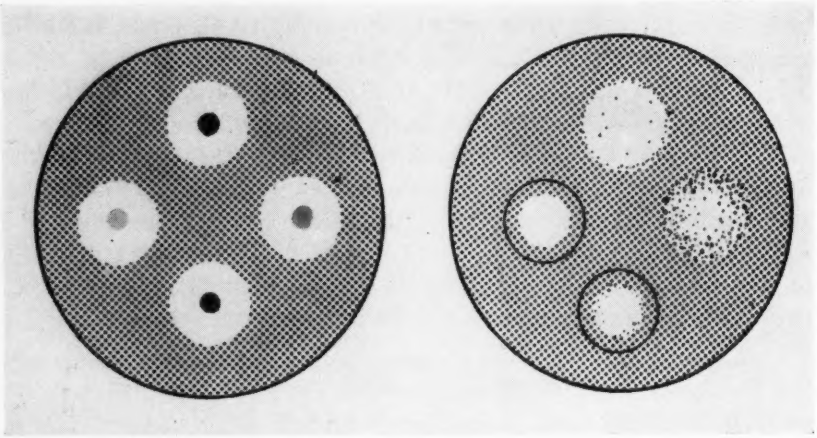

Fig. 1.—A primary plate (left) and its corresponding replica plate (right) are shown diagrammatically. Shading represents bacterial growth. The primary plate shows the usual clear zones of inhibition surrounding discs containing four different antibiotics. The different bactericidal effects are seen on the right. The uppermost zone is equal in size to that of the inhibition zone, and few organisms have survived (bactericidal effect +). The zone on the right shows numerous survivors (±). The other two zones show a satisfactory lethal effect, but are significantly smaller in diameter than the inhibition zones, indicating that the effect is produced only by relatively high concentrations of the drug.

Method.—"Primary" plates were dried at 37° C. for two hours and inoculated by flooding the surface with a young broth culture in which turbidity was just beginning to appear; to achieve this, some of the streptococcal cultures were incubated at 37° C. overnight, but two to four hours' incubation was sufficient for the other organisms tested. Excess fluid was pipetted off and the plates dried in the incubator for one-half hour. Inocula of this density produced just confluent growth after overnight incubation. Blotting paper discs (Ford 428 Mill) soaked in the antibiotics were placed on the surface (three or four to a plate), and the plates incubated at 37° C. for eighteen to twenty hours. Zones of inhibition developed around the discs in the usual way, and their diameters were measured. Replica sampling was carried out by pressing a disc of sterile velvet (mounted on a wooden backing) on the surface of the primary plate and then pressing it on the surface of a sterile nutrient agar plate, the "replica" plate, which was then incubated overnight. In this way a mass subculture is made of the whole of the surface of a primary plate, organisms being transferred from a plate containing antibiotics to an environment free of drugs, in which any viable bacteria are able to multiply (Fig. 1). Replica plates used for testing streptococci were enriched with glucose (1 per cent) and serum (5 per cent) and incubated for forty-eight hours.

Interpretation.—The method is designed to give an estimate of the extent to which antibiotic concentrations at and just above the inhibitory level are bactericidal. The considerations to be borne in mind when interpreting the findings on replica plates have already been discussed in detail in a previous paper[5]; it is sufficient to say here that on primary plates the organisms immediately next to a disc are exposed to a concentration of antibiotic about one-twentieth of that with which the disc was soaked. Outward diffusion occurs rapidly and at about four to six hours the boundary of the zone of inhibition becomes defined. During the remainder of the period of overnight incubation, organisms within this zone are exposed to antibiotic concentrations ranging from the minimum inhibitory level to three or four times as much. With the size of inoculum used, the degree of lethal action is assessed with sufficient accuracy for clinical purposes by comparing the size of the zone on the replica plate with that of the inhibition zone and by noting the colony density within it. We have arbitrarily graded the bactericidal effect as satisfactory (recorded +) when no more than a few colonies develop within the zone of inhibition (representing with our inoculum approximately 1 in 10,000 survival) and moderate (±) for more crowded discrete colonies. Semiconfluent or denser growth is taken to indicate no significant bactericidal effect (-). It may be found that a zone of satisfactory lethal action is produced by an antibiotic on a given organism, but that its diameter is 4 or 5 mm. less than that of the inhibition zone: this means that such an effect is exerted only by concentrations significantly higher than those which are inhibitory. From the clinical standpoint, treatment of such an infection by the drug might still be effective if a much higher concentration in the blood could be achieved by increased dosage or by slowing down the rate of excretion.

RESULTS AND DISCUSSION

The findings for each antibiotic are recorded in Tables I through VI. The degree of lethal action is indicated by the use of the notation described previously. The fourth line ("R") shows the number of organisms which were resistant in the conventional sense (inhibitory) when tested on the primary plate; no bactericidal action was therefore possible. Only forty-seven organisms were tested against penicillin and bacitracin, the eighteen strains of *Esch. coli* being omitted: the latter were the only strains tested against polymyxin.

Penicillin, Streptomycin, Bacitracin, and Polymyxin.—Most workers have found each of these antibiotics to be rapidly lethal in concentrations little above those needed to inhibit the growth of sensitive organisms ("Group I" drugs of Jawetz and his colleagues[8]), and it can be seen from Tables I through III that testing by the replica plating technique gives results substantially in agreement with this generalization. The only exceptions were the three strains of enterococci on which penicillin exerted a moderate lethal effect, and two other strains of this species with similar findings for Bacitracin.

An observation of some theoretical interest was that when the action of penicillin on enterococcal strains was tested, the replica plates often showed a ring of discrete bacterial growth immediately surrounding the site of the blotting paper disc, even though the remainder of the zone was free of colonies up to the periphery. The effect was not seen with the other species or with other drugs. It indicates that the lethal effect of the highest concentrations of penicillin was less than that of the lower ones in the intermediate zone. This agrees with the findings of Eagle and his co-workers[9, 10] that high concentrations of penicillin are less lethal than lower ones to enterococci. They

TABLE I. BACTERICIDAL ACTION OF PENICILLIN ON FORTY-SEVEN BACTERIAL STRAINS

EFFECT	STAPHYLOCOCCI	ENTEROCOCCI	HEMOLYTIC STREPTOCOCCI
+	22	7	10
±	0	3	0
-	0	0	0
R	5	0	0

TABLE II. BACTERICIDAL ACTION OF STREPTOMYCIN ON SIXTY-FIVE BACTERIAL STRAINS

EFFECT	STAPHYLOCOCCI	ENTEROCOCCI	HEMOLYTIC STREPTOCOCCI	*Esch. coli*
+	27	10	8	16
±	0	0	0	0
-	0	0	0	0
R	0	0	2	2

TABLE III. BACTERICIDAL ACTION OF BACITRACIN ON FORTY-SEVEN BACTERIAL STRAINS AND OF POLYMYXIN ON EIGHTEEN STRAINS OF *Esch. coli*

	BACITRACIN		POLYMYXIN	
EFFECT	STAPHYLOCOCCI	ENTEROCOCCI	HEMOLYTIC STREPTOCOCCI	*Esch. coli*
+	27	8	10	18
±	0	2	0	0
-	0	0	0	0
R	0	0	0	0

TABLE IV. BACTERICIDAL ACTION OF CHLORAMPHENICOL ON SIXTY-FIVE BACTERIAL STRAINS

EFFECT	STAPHYLOCOCCI	ENTEROCOCCI	HEMOLYTIC STREPTOCOCCI	*Esch. coli*
+	13	0	1	10
±	8	0	0	7
-	6	10	9	1
R	0	0	0	0

TABLE V. BACTERICIDAL ACTION OF AUREOMYCIN ON SIXTY-FIVE BACTERIAL STRAINS

EFFECT	STAPHYLOCOCCI	ENTEROCOCCI	HEMOLYTIC STREPTOCOCCI	*Esch. coli*
+	3, 5*	1, 6*	6*	11*
+	7	1	4	2*
-	12	2	0	5
R	0	0	0	0

*These strains showed the bactericidal effect only in zones much smaller than the inhibitory ones, i.e., concentrations significantly higher than inhibitory were needed to produce the effect.

TABLE VI. BACTERICIDAL ACTION OF TERRAMYCIN ON SIXTY-FIVE BACTERIAL STRAINS

EFFECT	STAPHYLOCOCCI	ENTEROCOCCI	HEMOLYTIC STREPTOCOCCI	*Esch. coli*
+	8	0	2	0
±	8	0	0	0
-	11	1	7	18
R	0	9	1	0

also found that initial exposure of a culture to such high concentrations has a protective action during its subsequent exposure to lower concentrations. These are in fact the conditions to which the organisms in the center of each diffusion system are subjected: an initial transient exposure to a high concentration (about 2,000 units per milliliter of penicillin in this case) followed by prolonged exposure to much lower concentrations.

Chloramphenicol, Aureomycin, and Terramycin.—It is clear from Tables IV, V, and VI that these three drugs do not possess the over-all bactericidal effect found in the four antibiotics described previously. Some correlation between species and susceptibility to the lethal action of chloramphenicol is evident; all but one of the strains of streptococci failed to show any material bactericidal effect, although a considerable lethal effect was produced on most of the staphylococcal and *Esch. coli* strains. These findings confirm those of Jawetz and his colleagues[11] for the action of chloramphenicol on nine strains of enterococci, and of Gray[12] relating to its action on one strain of *Esch. coli.* There is no correlation between species and lethal effect in the findings for Aureomycin and Terramycin. It may be said in general that most of the strains tested with these drugs showed moderate or no lethal effect, which agrees with the findings of other workers.[1, 13] Aureomycin produced a satisfactory lethal effect on about one-half of the bacterial strains but in nearly all of these the zone of this effect was much smaller than the inhibitory zone, showing that the effect was due to concentrations markedly higher than those producing inhibition. The maintenance in a patient of blood concentrations of a similar order by increased dosage would, with drugs such as Aureomycin, be attended with severe side-effects and would not in fact be attempted. The generalization that these drugs usually act in the body as bacteriostatic agents therefore remains valid.

It is clear from the findings discussed previously that results obtained with this method are consistent with those found by the more usual, but more laborious, viable counting methods. On the whole they confirm these classifications suggested by Jawetz and his colleagues[8] which divide antibiotics into two groups. The first group is mainly bactericidal in action and includes the first four drugs mentioned previously, together with a few others; the second is mainly bacteriostatic: chloramphenicol, Aureomycin, and Terramycin being found in this group. There is, however, variation from strain to strain within a given species in response to an antibiotic and the result cannot be accurately forecast on general grounds. It therefore seems likely that the simple test described here may be of value in routine bacteriologic work as an adjunct to the usual form of "sensitivity test." A report on an organism might be made in the following terms: "sensitive to penicillin; bactericidal action +," or "sensitive to chloramphenicol; bactericidal action at inhibitory level unsatisfactory." The true value of the technique can be assessed only when such results can be compared with the results of therapy.

SUMMARY

A new method is described for the assessment of the bactericidal action of drugs in vitro. It may conveniently be carried out as an extension of the blotting paper disc type of "sensitivity test."

With its aid, tests were made of the bactericidal action of seven antibiotics on sixty-five bacterial strains representative of several pathogenic species. Results with this technique were broadly in agreement with those found by other methods. Inhibitory concentrations of penicillin, streptomycin, polymyxin, and bacitracin were in general highly lethal; chloramphenicol, Aureomycin, and Terramycin were predominantly bacteriostatic, but in high concentration were lethal for some of the strains tested. The number of exceptions found, however, appears sufficient to justify use of the method in routine work.

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VAGINAL AND URINARY SEDIMENT SMEAR STAINING TECHNIQUE WITHOUT PREVIOUS FIXATION

Adapted to Papanicolaou's and Shorr's Staining Methods

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TRIAL and development of new methods and stains are desirable for progress in cytology. Therefore, if the vaginal smear techniques could be rendered easier and more labor saving, it would be of practical interest because of the importance of these procedures in the study of the sexual function and genital and urinary cancer.

One of the authors (L. J. L.) has pointed out in a previous comparative study[1] that the results obtained using a special technique for dried cytologic material furnished results similar to those of Shorr's original procedure in material previously fixed in alcohol-ether. This technique was suggested from the procedures of Kiserud and associates[2] who have developed a tetrachromic and a multichromic stain suitable for routine use in cytologic dried material.

The fundamental principles involved in Kiserud's methods made advisable their application to other more common staining techniques, such as those of Shorr and Papanicolaou, because these principles facilitated the transportation of the smears to the examiner and, in addition, the fixation step may be omitted using these methods. As other investigators have stated, more errors are made as a result of faulty or delayed fixation than in any other single step of the staining treatment.

In our use of the methods of Shorr and Papanicolaou over a period of time, using smears with dried material, we have introduced certain modifications derived to a great extent from the procedures of Kiserud and co-workers, which seem sufficiently important to warrant their being reported, taking into account the widespread interest in these determinations which resulted in their general clinical use.

It is the primary purpose of this paper to compare the results obtained by two classical methods with those given by the same procedures modified to obtain satisfactory stained smears in dried material.

MATERIAL AND METHODS

This study was carried out on ten normal women who did not present signs or symptoms of any functional abnormality. Vaginal smears were taken at about mid-cycle when estrogen concentration was supposed to be higher.

All preparations were made according to the recommendations of Allende and Orías[3] and Di Paola.[4] The bulb aspiration tube was used to collect mucus from the posterior fornix prior to examination or instrumentation. Spreads of the vaginal mucus were made immediately on glass slides.

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TABLE I. VALUES OF SUPERFICIAL ACIDOPHILIC CELLS IN VAGINAL AND IN URINARY SEDIMENT SMEARS (CORRECTED BY ANGULAR TRANSFORMATION)

CASE NO.	VAGINA								URINARY SEDIMENT							
	FIRST DAY			FIFTH DAY			TWENTY-FIFTH DAY		FIRST DAY			FIFTH DAY			TWENTY-FIFTH DAY	
	SHORR FIXED	SHORR DRY	TETRAC.	SHORR FIXED	SHORR DRY	TETRAC.	SHORR FIXED	SHORR DRY	TETRAC.	SHORR FIXED	SHORR DRY	TETRAC.	SHORR FIXED	SHORR DRY	TETRAC.	SHORR FIXED	SHORR DRY	TETRAC.
1	33.21	33.46	33.27	34.27	33.09	31.63	30.66	27.97	35.67	21.97	23.58	18.91	22.79	19.37	21.13	27.97	23.58	20.27
2	22.71	24.80	23.42	15.12	19.64	20.96	22.22	27.97	27.76	20.27	21.13	18.44	19.37	21.13	20.27	21.97	17.46	19.37
3	24.80	21.97	25.10	23.97	22.63	25.84	27.69	24.65	29.60	27.97	27.28	27.28	27.63	28.25	26.06	26.13	28.73	27.06
4	9.98	10.94	11.24	9.98	12.39	17.95	18.44	22.71	19.64	7.71	8.13	7.92	9.81	16.32	14.77	14.18	19.28	9.46
5	24.73	24.35	26.71	24.95	23.34	25.77	22.30	20.53	23.56	26.85	26.28	23.58	25.70	23.58	20.27	26.13	27.49	22.55
6	19.37	18.44	20.70	20.88	22.30	23.89	21.22	23.50	21.39	14.18	17.46	16.43	14.18	14.18	15.34	15.34	14.18	14.18
7	22.38	21.97	22.14	22.38	20.00	21.97	13.69	24.73	17.85	20.79	22.46	21.81	20.79	21.47	23.42	19.73	18.24	21.64
8	23.34	20.36	20.79	23.81	23.34	30.46	17.16	20.00	22.06	22.79	21.13	21.13	22.22	22.46	22.79	21.81	18.63	23.11
9	26.13	24.43	25.48	26.56	22.55	21.05	23.26	27.83	26.64	23.26	24.27	19.37	24.73	23.58	22.63	20.36	17.76	21.22
10	28.66	32.20	32.52	27.28	27.97	30.26	28.93	31.69	30.59	25.55	24.58	27.56	23.81	26.42	24.80	24.73	27.69	26.42
$\bar{x}$	23.53	23.29	24.13	22.92	22.72	24.97	22.55	25.15	25.47	21.13	21.63	20.24	21.10	21.67	21.14	21.83	21.30	20.52

$\bar{x}$ = mean.
Tetrac.: tetrachromic stain.

We obtained nine specimens from the same patient and immediately placed three of them in a mixture of alcohol-ether to be stained at intervals of one, five, and twenty-five days. Two specimens were kept at room temperature for twenty-four hours; two for five days; and the remaining two for twenty-five days, in order to evaluate accurately the influence of the time interval between the collection of the sample and the staining treatment.

Urinary sediment smears were carried out simultaneously, using the first urine voided the morning that the vaginal smears were made. The method used was that of Del Castillo and associates[5]; the estimations were made on dried and fixed material using the same procedure as with the spreads of vaginal mucus.

We obtained two vaginal smears from each patient of another group of ten patients. One smear was treated and stained according to the classical procedure of Papanicolaou[6] and the other was allowed to dry in the air without fixing it in alcohol-ether, to be treated afterward by the same method modified.

All the material fixed in alcohol-ether was stained by Shorr's[7] or Papanicolaou's[6] original staining methods. For the dried material we employed the tetrachromic stain of Kiserud and associates[2] and the following modification of Shorr's and Papanicolaou's staining procedures: (1) Leave under running tap water, two minutes; (2) Place in acetic acid 2 per cent alcoholic solution (30 per cent alcohol), two minutes; (3) Acetic acid 2 per cent alcoholic solution (70 per cent alcohol), two minutes; (4) Stain.

A. *Papanicolaou:* Run the original staining procedure as usual until mounting.

B. *Shorr:* Stain for four minutes; (5) Wash in tap water; (6) Dehydrate, rinsing with acetic acid 2 per cent alcoholic solution (70 per cent alcohol); (7) Dehydrate, rinsing with acetic acid 2 per cent alcoholic solution (95 per cent alcohol); (8) Dehydrate in absolute alcohol; (9) Clear in xylol and mount in balsam.

The cellular classification was the one suggested by Di Paola,[4] based on staining affinity without referring to the cornification process. The cells were classified as superficial acidophilic cells, superficial basophilic cells, intermediate basophilic cells, and profound basophilic cells.

We have adopted a system which seems concise, simple, and yet sufficiently flexible to fit into the framework of a wider comparative plan; it also fulfills the requirements necessary to support a valid statistical control.

Since disagreement concerning the interpretation of smears stained with different techniques at diverse intervals was considered possible, and because we were particularly interested to learn whether the lack of fixation would be reflected in tinctorial or morphologic changes, we submitted all the results obtained to statistical analysis.

RESULTS

The figures of superficial acidophilic cells (cornified) were selected for statistical analysis since they represent the most valuable index of estrogenic activity. We worked with the original mean values (expressed in percentages) corrected by angular transformation. These figures are presented in Table I.

From the beginning we observed that there was a clear correlation between the figures for superficial acidophilic cells obtained by the different procedures carried out at the same time. In an attempt to confirm this impression and to isolate the sources of variations from those stemming from the method under test, the data were submitted to an analysis of variance (Table II).

It may be seen that the F for time (.391) and for "methods" (.548) were not significant. The assay demonstrated that the smears did not show any

TABLE II. ANALYSIS OF VARIANCE

SOURCES OF VARIATION	DEGREES OF FREEDOM	Sx^2	MS	F
Time	2	7.77	3.88	0.391
Methods	2	10.88	5.44	0.548
Cases	9	3182.17	353.57	35.54
Source (vagina-urine)	1	323.66	323.66	32.63
Experimental error	165	1637.42	9.92	

Sx^2: sum of squares, MS: mean square, F: variance ratio.

important change derived from these factors, the percentage of superficial acidophilic cells being the same with both procedures. They also indicate that the period imposed by delaying the staining treatment for one, five, or twenty-five days had no effect on the efficiency of the methods in regard to the morphologic and tinctorial characteristics of the cells.

The analysis also shows that there is a significant F for "source of sample" (32.63) and for "cases" (35.54) indicating that although the levels of acidophilic cells are different in vagina and urine, their response to different conditions ("cases") is equally sensitive, as it has been demonstrated in a previous report by one of the authors (L. J. L.).[8]

A statistical evaluation of the modified Papanicolaou stain technique has not been described, but microscopic observation disclosed that the cytologic structural details are preserved. This has been confirmed by pathologists with great experience in this type of study; at times they were unable to discover any difference in the tinctorial or morphologic characteristics among smears taken simultaneously from the same patient and treated at different times by various staining procedures. Results of the statistical analysis of 200 determinations made on fifty patients and normal subjects are to be presented in a separate communication.

COMMENTS

The choice of a method for a particular purpose will depend on a variety of factors, such as the specificity and precision required, the laboratory facilities, and the nature of the information desired.

From the point of view of this communication, the fact to be emphasized is that the methods we advocate will contribute valuable diagnostic procedures to general practitioners and to centers without laboratory facilities. These methods simplify the handling and transporting of the specimen before it is delivered to the specialized examiner. The time interval between the collection of the sample and the staining treatment does not affect the precision and the specificity of the procedures.

Other advantages of the method are that it usually permits a larger number of comparisons to be made; requires less special equipment for its use (fixation liquids are eliminated); and the possibilities of losing material in the manipulation are diminished.

The fixation step, which represents a potential loss of material due to dispersion, is eliminated. This is particularly important for those samples with

a small quantity of material, because it must be remembered that the smears obtained from the filtrate of urine retain a very high degree of humidity.

Evidence compiled in our study clearly indicates a statistically significant correlation between the classical procedure of Shorr and Papanicolaou using material previously fixed in alcohol-ether, as compared with the same methods modified to be applied to dried samples.

In our endeavor we have been guided by the desire to stimulate and emphasize the growing importance of the cytologic approach and we hope that the results described will contribute to increasing the use of these determinations.

SUMMARY

A modification is suggested to adapt the stain techniques of Shorr and Papanicolaou in order to make satisfactorily stained vaginal and urinary smears in dried material.

The statistical analysis of the data obtained proves that there is a significant positive correlation between the original and the modified procedures. This is evidenced by the fact that the number, size, staining properties, and morphologic characteristics of the cells are preserved.

This correlation was not affected by the interval between the time the material was obtained and the time it was stained, the tinctorial and structural characteristics of the cells being the same with any of the procedures employed.

The advantages with respect to proper management of the material and convenience are described, and it is felt that the fundamental principles in the modification proposed are sufficiently simple to warrant its wider use as a valuable adjunct to other biologic diagnostic procedures.

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A RAPID COLORIMETRIC METHOD FOR THE DETERMINATION OF ISONICOTINIC ACID HYDRAZIDE IN BLOOD PLASMA

II. Application to Blood Plasma From Human Tuberculosis Patients

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THE treatment of human tuberculosis with isonicotinic acid hydrazide (INH) has made essential the study of methods for the determination of this compound in the blood and body fluids. Two chemical methods now in use were developed by Kelly and Poet,[1] and Rubin and associates.[2] Both methods involved the use of volatile toxic reagents (isoamyl alcohol-ether mixture and cyanogen bromide, respectively), and required several manipulations (extraction, oxidation, hydrolysis, etc.). Strickland and Hentel[3] reported a gasometric determination of INH in terms of the amount of nitrogen released from the compound in the presence of alkaline potassium iodate. This method is not affected by the presence of p-aminosalicylic acid (PAS) in the serum as are other methods. More recently, another colorimetric method has been reported by Jacobs[4] for measuring the reduction of potassium ferricyanide in acid solution by isoniazid. A rapid colorimetric technique with less hazardous reagents has been reported from this laboratory to measure quantitatively with a pyridine derivative as indicator the levels of isoniazid in protein-free filtrates from plasma of laboratory animals experimentally treated with the drug (Prescott, Kauffmann, and James[5]). The present report concerns the application of the "pyridyl" method to plasma from tuberculous patients under treatment with isoniazid.

METHOD

"Pyridyl" Method.—The test blood is prevented from clotting by the use of ACD,* and the plasma separated by centrifugation. A 2 ml. sample of the plasma is diluted with 6 ml. of distilled water and deproteinized with 2 ml. of 15 per cent trichloroacetic acid solution. Aliquot parts of the protein-free filtrate are then assayed for INH by the following technique: to 2 ml. of filtrate in a 5 ml. volumetric flask is added 0.5 ml. of a 1 per cent aqueous solution of 4-pyridylpyridinium dichloride reagent (Eastman Kodak product) followed by 0.7 ml. of 2-normal sodium hydroxide and 0.5 ml. of 2-normal hydrochloric acid. After thorough mixing, the volume is adjusted to 5 ml. with distilled water, and the mixture held at room temperature for fifteen minutes. Transferring to microcells, colorimetric readings are then made against distilled water in a Fisher electrophotometer

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*ACD contains 8 Gm. citric acid, 22 Gm. sodium citrate, 24 Gm. dextrose in 1,000 ml. water; .5 ml. of this solution is added to 5 ml. blood.

with a 425 mμ filter. A predosage control plasma determination is necessary to correct for nonspecific color. For since the "pyridyl" indicator gives color with p-aminosalicylic acid and the sulfonamides, even a slight trace of either of these chemicals in the plasma would increase the result. In addition, a standard INH solution is used with all plasma determinations.

RESULTS

The results obtained from determining plasma levels in twenty-four tuberculous patients selected at random showed a wide variation even with a constant dose of 200 mg. orally and a constant time for obtaining the blood sample, two hours after drug administration. Fig. 1 shows that the plasma levels, corrected for individual blanks, ranged from a high of 1.40 mg. per cent (14 μg per milliliter) to as little as 0.10 mg. per cent (1 μg per milliliter) and averaged 0.40 mg. per cent (4.0 μg per milliliter). Also on this figure may be seen a similar wide variation of the two-hour plasma levels in a group of nine patients, who had been given a 100 mg. oral dose, in whom the levels ranged from 1.04 mg. to 0.2 mg. per cent. It is of interest that the average of this small group is 0.44 mg. per cent and thus closely similar to the 0.40 mg. average following the larger dose.

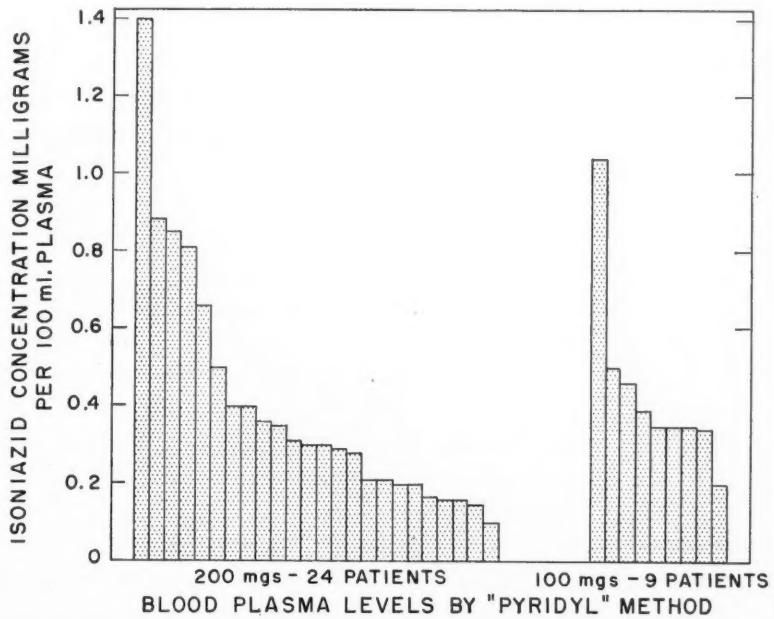

Fig. 1.—Individual plasma levels two hours after oral dose of INH in tuberculous patients.

Comparison of Three Methods of Assay.—Inasmuch as the "pyridyl" color test is a direct reaction between unaltered hydrazide and the indicator, it was felt desirable to compare the test with the Kelly and Poet method and the Rubin technique, each of which depends upon a split product of the hydrazide:

hydrazine, after hydrolysis in the Kelly and Poet method, and isonicotinic acid, by oxidation in the Rubin method. Table I shows the results of the comparative analyses on fourteen available human plasma samples by the Rubin

TABLE I. COMPARISON OF INH PLASMA LEVELS IN TUBERCULOUS PATIENTS BY THREE METHODS OF ASSAY

PATIENT	PLASMA LEVEL 2 HOURS AFTER A SINGLE 200 MG. ORAL DOSE (MG. PER CENT) METHOD		
	"PYRIDYL"	RUBIN	KELLY-POET
H. H.	0.31	0.11	0.13
C. M.	0.30	0.11	0.10
A. T.	0.21	0.06	0.17
L. S.	0.29	0.11	0.16
O. B.	0.21	0.21	0.22
A. F.	0.16	0.20	0.14
H. P.	0.10	0.12	0.10
R. W.	0.35	0.35	0.31
D. W.	0.40	0.35	-*
O. T.	0.16	0.20	-
W. S.	0.18	0.27	-
P. D.	0.36	0.40	-
R. M.	0.40	0.44	-
W. E.	0.39	0.42	-
Range	0.10-0.40	0.06-0.44	0.10-0.31

*"-" indicates not done.

method and eight plasma samples by the Kelly and Poet method. All of these tuberculous patients were given 200 mg. of INH orally, and blood assays were performed two hours later. These methods were also found to give nonspecific color in predosage plasma controls. Hence the results reported are corrected for this nonspecific color. From this experiment it may be inferred that all three methods give closely similar results on plasma from tuberculous patients under treatment with INH only.

It should be pointed out that the pyridyl method will give color with free aromatic amines, and therefore in some individuals after treatment the plasma INH level may be increased if a metabolite with such chromogenic structure is present. It is significant that the pyridyl reagent does not combine with the acetylated derivative of INH, which is nonbacteriostatic, and hence the assay is a measure of the therapeutically active, unaltered INH.

Study of the Rate of Absorption of INH.—One experiment is included on the determination of plasma levels at different time intervals after a single dose of INH to show the rate of absorption of the drug. Plasma from five tuberculous patients was tested immediately before a 100 mg. oral dose of INH (average 2.8 mg. per kilogram), and then again one, two, three, twelve, and twenty-four hours after the drug administration. Fig. 2 shows a peak level of 0.46 mg. per cent within one hour in one case and between 1.04 mg. per cent and 0.34 mg. per cent at two hours in the other four individuals. There is then a gradual decrease in the blood level in all cases with little or no drug remaining twenty-four hours after administration. This curve is thus similar to those reported by

Rubin[2] and by Elmendorf and associates[6] (Kelly and Poet method of assay), each of whom had employed a dose of approximately 3 mg. per kilogram in six patients.

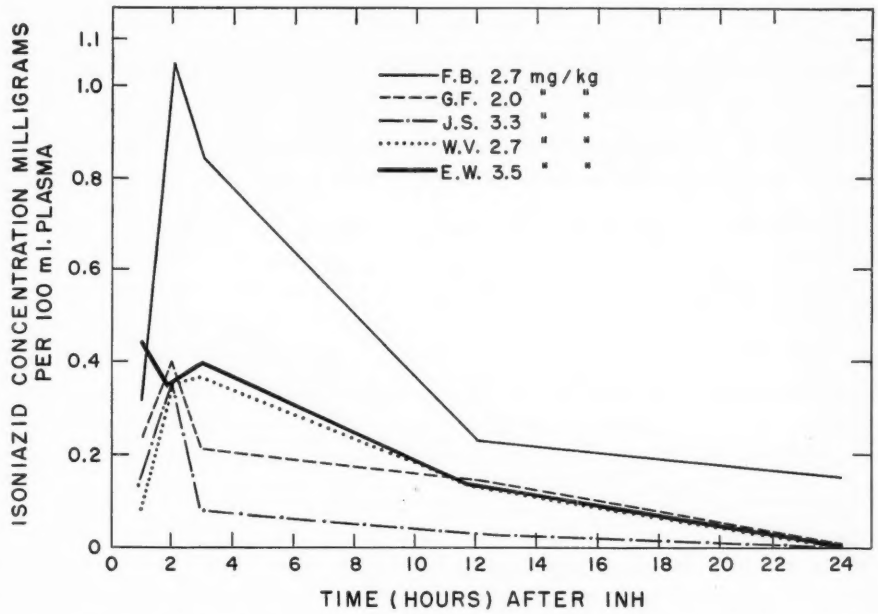

Fig. 2.—Rate of absorption of 100 mg. dose of INH in five tuberculous patients. Blood plasma levels by "pyridyl" method.

SUMMARY

A method of assay of INH employing 4-pyridylpyridinium dichloride as indicator has been applied to plasma from tuberculous patients. Plasma levels two hours after INH administration in thirty-three tuberculous patients, assayed by this method and corrected for chromogen, showed wide individual variation. Study of the rate of absorption of INH in five patients showed that a peak plasma level was reached within two hours. The results indicate that the method provides a simple and rapid procedure for determination of isoniazid in blood plasma from tuberculous patients.

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LABORATORY METHODS

MAINTAINING STERILITY DURING STORAGE OF ARTERIAL HOMOGRAFTS

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THAT preserved segments of aorta or major arteries which have been removed from suitable donors within a few hours after death may be used as arterial grafts is well established.[1-2] These grafts must be sterile at the time of implantation, or abscess formation, necrosis, and hemorrhage are likely to occur. Even if fatal hemorrhage were avoided and infection controlled, subsequent fibrosis would be likely to cause constrictive narrowing of the graft.

To assure sterility of a homograft, certain precautions are necessary:

First, the graft must be sterile when it is placed in storage. Most arterial banks[3-5] use only grafts that have been obtained aseptically, within a few hours after death, from donors who gave no evidence of bacterial infection or contamination of the aorta or blood stream. Several methods of sterilizing grafts obtained under less favorable circumstances have been suggested and utilized experimentally.[6-8] At present, however, these methods either are not practical for general use, have not proved entirely effective, or have been injurious to the graft in a way that has impaired its elasticity and durability or has made it a factor predisposing to thrombosis.

Second, the graft must be preserved from contamination during the storage period. If the nutrient medium-refrigeration method of Gross[1] is utilized, occasional cultures of the storage medium can be made to test its sterility, but even these procedures themselves present the possibility of introduction of contaminants. Since the storage medium is an efficient culture medium also, a few contaminants that might escape detection at one time could reproduce and result in severe contamination after several days or weeks of storage. Even in the freezer or lyophilization methods of storage, viable organisms could be introduced during the freezing or thawing processes, or a few undetected contaminants, present when the graft was frozen, could survive the freezing process and, thawing, could multiply and cause infection.

Because of these considerations, it has become standard practice in most arterial banks,[3-5] to place an antibacterial agent in the solution in which the

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graft is washed when it is first obtained and also in the storage medium. The usual agents are penicillin, 100 units per cubic centimeter, and streptomycin, 0.1 mg. per 100 ml.

OBJECTIONS TO USE OF PENICILLIN AND STREPTOMYCIN

Although we are not aware of reports of unfavorable effects from use of these antibiotics, certain objections to their use might be raised:

First, neither penicillin nor streptomycin is fully effective against all organisms; the same is true of the two when used together. Certain bacteria, such as those of the genus Pseudomonas and those of the genus Proteus, are relatively resistant to these preparations. Growth of viruses and fungi is not significantly inhibited by them. Four of our first twelve homografts, preserved in media containing penicillin and streptomycin, contained grossly visible fungi after three to six weeks of storage by the nutrient medium-refrigeration method. Others[2, 9] have reported similar experiences.

Second, both penicillin and streptomycin deteriorate rather rapidly in solutions of the concentrations commonly used. This is particularly true when the storage medium contains biologic material, such as serum, or viable tissue, such as a segment of aorta, either of which may contain enzyme systems capable of interfering with the effectiveness of antibiotics. Probably little, if any, antibiotic effect from penicillin or streptomycin is maintained in homograft media after a week or more of storage.

Third, the rather rapid and continuing emergence of strains of common contaminating bacteria which are resistant to penicillin and streptomycin, either or both, is a source of concern, since contamination of the graft with such organisms would not be eradicated by these antibiotics in the storage medium. Furthermore, a clinical infection resulting from implantation of such a contaminated graft would not respond to administration of these antibiotics. This objection alone seems to us a potent argument against use of penicillin and streptomycin as bacterial inhibitors in arterial homograft banks.

THEORETIC REQUIREMENTS OF A SATISFACTORY ANTI-INFECTANT FOR USE IN ARTERIAL BANKS

Assuming that use of some decontaminant in solutions for washing, débridement, and preservation of arterial homografts is indicated, we suggest the following theoretic requirements for acceptance of such an agent:

1. The agent should be effective against all contaminants, including bacteria, fungi, and viruses.

2. The agent must not lose significant potency by storage in the preservation medium, in the presence of a viable arterial graft, and when subjected to the conditions under which the artery bank operates (freezing, refrigeration, or lyophilization).

3. The agent must be innocuous to the arterial graft, in no way damaging its physical properties or affecting it in any manner which would predispose to thrombosis or deterioration when the graft is implanted in a recipient.

MERTHIOLATE AS AN ACCEPTABLE ANTI-INFECTANT

Published reports[10, 11] indicate that a dilute, aqueous solution of sodium ethylmercurithiosalicylate (Merthiolate) might prove an acceptable agent for maintaining sterility in arterial homograft banks. In dilutions of 1:10,000 this compound has been used successfully in the preservation of plasma and other biologic materials.[10, 11] It is reported[10] to kill or inhibit growth of most

known contaminants; evidently it does not deteriorate perceptibly on standing, and in the recommended concentrations it does not seem injurious to human tissues.

In order to test the ability of Merthiolate to meet the theoretic requirements listed in the preceding section of this paper, we began to use this agent in our experimental arterial homograft bank; the subjects were dogs.

For this use, 1 Gm. of Merthiolate* crystals and 1.4 Gm. of powdered sodium borate were dissolved in 50 ml. of distilled water, giving a buffered stock solution containing Merthiolate in a concentration of 20 mg. per milliliter. By adding 5 ml. of this stock solution to 95 ml. of any desired solution, a concentration of Merthiolate of 1:1,000 (or 1 mg. of Merthiolate per milliliter of solution) can be obtained. This concentration of Merthiolate (1:1,000) was used in simple studies carried out to answer the following question:

1. *Is Merthiolate in concentrations of 1:1,000 effective as an anti-infectant for solutions used in arterial graft banks?* First, we prepared storage medium by adding fresh human serum to Ringer's solution (10 per cent serum by volume). Although the solution and serum used were originally sterile, no attempts were made to maintain sterility. The solution was placed in three glass storage bottles, each of a capacity of 8 fluid ounces, approximately 240 ml., which had been washed in tap water but were not sterile. After these solutions had stood for eight hours, cultures were made from them. In each instance, 30 ml. of Bactofluid thioglycolate medium in a tall tube was inoculated with 0.1 ml. of the test solution; also inoculated was a blood-agar plate and an eosin-methylene blue plate. These cultures were incubated for forty-eight hours at 37° C. Micrococci and gram-negative bacilli of the coli-aerogenes group were recovered from all of the solutions. Merthiolate from the stock solution was then added to each container of contaminated nutrient media to produce a final concentration of 1:1,000. After twenty-four hours the solutions were cultured again; all gave negative results at the end of forty-eight hours of incubation. This indicated to us that Merthiolate in a concentration of 1:1,000 will effectively control incidental contamination of nutrient medium used in arterial graft banks.

Next we took aortic grafts from three dogs which had been dead less than six hours. A clean, but not aseptic, technique was used and in each instance the saline solution in which the fresh grafts were washed prior to storage was found to be contaminated bacteriologically. These grafts, also contaminated, were placed in sterile nutrient media containing Merthiolate, 1:1,000, and stored in the usual manner at 2° to 4° C. After eight hours, the medium in which each of the grafts was stored was cultured according to the procedures just described; one was found to be sterile and two contaminated. After twenty-four hours of storage the media in which all three grafts were stored were sterile and repeated cultures in the course of the subsequent three weeks were persistently negative. This seems to indicate that Merthiolate, 1:1,000, is capable of controlling contamination which may occur in the course of procurement and preparation of arterial grafts for storage.

Evidence of the efficacy of Merthiolate in maintaining sterility of arterial grafts during storage by the nutrient medium-refrigeration method, under actual clinical conditions, is derived from further observations. There was no growth in cultures taken during the period of storage of aortic grafts, nine from human beings and six from dogs, taken under aseptic conditions and stored for from five days to eight weeks. At least two tests of sterility were made during the storage period of each graft and, in relation to several of the grafts, there were as many as six negative cultures.

2. *Is Merthiolate (1:1,000) injurious to blood vessel grafts?* Merthiolate was added to isotonic saline solution in which segments of external jugular vein were washed and kept moist for from thirty to sixty minutes during the operation of transplanting these segments

*Kindly supplied by Eli Lilly & Company.

of jugular vein, as fresh, autogenous venous grafts, to the abdominal aortas of dogs. Fifteen such fresh venous autografts were exposed to a 1:1,000 solution of Merthiolate for thirty to sixty minutes before implantation in the aorta. All grafts "took" perfectly. There was no leakage and no thrombosis. Furthermore, there was no increase in morbidity and no recognizable increase in tissue reaction around the grafts, as compared with experiences in which Merthiolate was not used. All grafts were patent and functioning well when studied by aortography three to six months after implantation.

Next, we added Merthiolate to the storage medium (composed of Ringer's solution and 10 per cent fresh serum) in which aortic homografts from dogs were stored. Two aortic grafts stored in media containing Merthiolate, 1:1,000, refrigerated at 2 to 4° C. for three and five weeks, respectively, were implanted as homografts in the abdominal aortas of dogs. These grafts appeared to have excellent physical properties at the time of implantation and both functioned well, without complications, for a period of six months after operation, during which observation continued. Similarly, we added Merthiolate, 1:1,000, to the saline solution in which two aortic grafts from dogs were washed prior to their preservation, and also to the serum in which they were stored two and three months, respectively, by the freezer method at -80° C. There were no recognizable detrimental effects on the grafts, and results after surgical implantation were entirely satisfactory.

After these favorable results we began using Merthiolate instead of penicillin and streptomycin in our human arterial bank. To date, nine aortic homografts preserved in solutions containing Merthiolate, 1:1,000, have been implanted in human patients (seven grafts were stored by the nutrient medium-refrigeration method and two by the freezer method). All these grafts had excellent physical properties at the time of implantation and there were no complications attributable to the grafts during the early (three weeks to three months) postoperative period during which observation was maintained.

LIMITATIONS OF MERTHIOLATE AS A PRESERVATIVE FOR USE IN ARTERIAL BANKS

It seems evident that in proper concentration, Merthiolate, placed in the wash saline solution and in storage medium used in arterial banks, is capable of controlling incidental contamination while not injuring arterial grafts. The most desirable concentration for this use has not been determined. Concentrations which are too high, however, may well prove injurious, predisposing to thrombosis or impairing the physical properties of the grafts. It seems reasonable on the other hand, to use as high a concentration as possible without injuring the grafts until equal efficiency has been demonstrated for lower concentrations.

Another factor of importance is the extent or severity of contamination that can be controlled by this agent. In all our studies, relatively mild contaminations were tested. We do not advocate abandoning aseptic technique in the procurement and preparation of grafts. We do not believe addition of Merthiolate or of any other chemical decontaminant to the wash saline solution or storage medium will effectively sterilize grossly contaminated grafts in any manner comparable to exposure to high-voltage cathode rays, as reported by Meeker and Gross, nor should it be expected to do so. In this respect we feel obliged to report our experience with a human aortic graft taken aseptically two hours after death. The donor had died with unrecognized

bacteremia and meningitis. Cultures of the saline solution in which the fresh aorta was washed were positive for streptococci. The graft was stored in nutrient medium containing Merthiolate, 1:1,000. Cultures were obtained after twenty-four hours, again after three days, and finally after two weeks, of storage at 2° to 4° C.; from all, these same organisms were obtained. We assume that blood-borne bacteria lodged in the vasa vasorum, the capillaries, and perhaps in the tissue fluids of the graft were so numerous, or that the organisms were so well protected within the tissue, that the Merthiolate in the storage medium was incapable of sterilizing the graft. Obviously this graft was never considered fit for surgical implantation and was discarded.

SUMMARY

We found a buffered solution of Merthiolate, in a concentration of 1:1,000, to be a safe, efficient, and satisfactory decontaminating agent for use in solutions employed in arterial homograft banks. For this purpose it had certain theoretic and practical advantages over the more commonly used penicillin and streptomycin. However, it could not be relied on to sterilize grossly contaminated arterial segments; rather, it was used in a prophylactic manner, to maintain sterility of grafts obtained under the most favorable, aseptic conditions.

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THE PLATE COMPLEMENT FIXATION TEST WITH LEPTOSPIRAL ANTIGENS

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SINCE the preparation of leptospiral antigens for complement fixation tests[1-4] is rather laborious, any procedure that would conserve materials would be of considerable advantage. The plate complement fixation test described by Fulton and Dumbell[5] using allantoic fluid infected with influenza virus and later shown to be practical with other viral antigens[6-9] appeared to be applicable to serologic studies with leptospiral antigens. This investigation was undertaken to study the adaptability of this procedure to serologic work with leptospirae.

MATERIALS AND METHODS

Antigens.—The antigenic preparation used most extensively was a washed suspension of leptospirae from culture. Korthof's medium[10] in 100 ml. amounts was seeded with 1/10 vol. of actively growing culture. The leptospirae were harvested after five to seven days' growth at room temperature on the assumption that antigenic material might be lost into the culture fluid if incubation were prolonged.[4] Merthiolate to a concentration of 1:10,000 was added and the leptospirae sedimented at about 20,000 X gravity (Spinco) for thirty minutes. After washing three times in isotonic phosphate saline solution,[11] the suspension was standardized to 45 per cent transmission in a Lumetron photoelectric colorimeter using a 14 mm. tube and an M 420 filter. These standardized suspensions of various types of leptospirae represented 1/10 to 1/7 of the original volume of culture and contained approximately 5×10^8 organisms per milliliter. The second type of antigen used was a sodium hydroxide extract prepared as previously described,[4] except that washed leptospiral suspensions from cultures instead of infected allantoic fluids were extracted. Each milliliter of the neutralized extract represented about 2×10^9 organisms.

Technique of the Complement Fixation Test.—The procedure in general followed that previously employed with viral antigens,[5, 7, 9, 12] with certain minor modifications. Plexiglas plates 1/4 inch thick and 12 inches square with 100 one-inch squares marked on the surface were used. Drops were distributed to the squares by means of 19 gauge needles[7] fitted to Wintrobe pipettes. When the tips of the needles were lightly lubricated with petroleum jelly, drops of 20 cu. mm. were consistently delivered. Without lubrication the drops tended to be larger and varied in size according to the angle at which the needle was held and the surface tension of the fluid. The cabinets in which the plates were incubated were modeled after those described by Morgan and associates,[12] but were constructed of plywood and sealed inside and out with several coats of enamel paint. Drops tended to evaporate during incubation until holes were bored in the margins of the plates to provide adequate circulation. Fixation at 37° C. for two hours was tried but was replaced by fixation overnight at 50° C. when it was found that reactions under the latter conditions were significantly more sensitive. It was also found that thirty minutes at 37° C. was insufficient time for complete hemolysis to occur with minimal amounts of complement. Consequently, a period of two hours was used. The plates were then returned to the cold cabinet for one hour before reading in order to facilitate the formation

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of buttons. All dilutions were made in Veronal buffer solution.[13] The hemolytic system consisted of equal parts of 0.4 per cent sheep cells and a dilution of hemolysin to give maximal sensitization.

RESULTS

Reactivity of Antigens.—In order to determine the optimal concentration of each antigen, dilutions of standardized suspensions were tested against varying amounts of complement and doubled dilutions of homologous immune rabbit serum. Thus, on one plate the amount of complement fixed by a given antigen dilution in the presence of nine concentrations of immune serum and the amount fixed by antigen alone could be determined. Complement was

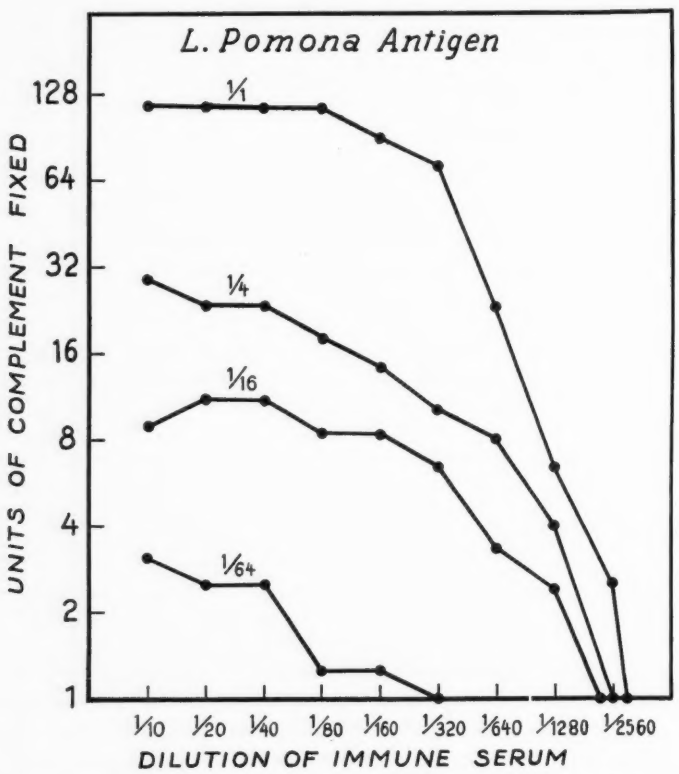

Fig. 1.—Titration showing the amount of complement fixed in relation to the concentrations of antigen and rabbit immune serum.

diluted to provide a series with a dilution factor of 1.6. The unit of complement was usually between 0.08 and 0.16 cu. mm. The results of such a titration are shown in Fig. 1. To determine the amount of complement fixed specifically, the amount fixed by antigen alone (serum usually was not anticomplementary) was subtracted from the amount fixed by antigen and serum and the difference was expressed in units as determined in diluent alone. Fig. 1 shows that the undiluted standard suspension did not contain an excess of antigen for low dilutions of serum. The amount of complement fixed in the

presence of an excess of antibody was roughly proportional to the concentration of antigen. Decreasing the amount of antigen, however, over a certain range caused very little reduction in the maximal serum titer. Twenty cubic millimeters of a 1:8 dilution of the standard suspensions was chosen as the antigenic dose, since this amount was in excess of the amount required to give maximal serum titer and since higher concentrations of antigen tended to be anticomplementary. This dose of antigen prepared from various types fixed from 13 to 25 units of complement in the presence of 1:10 dilutions of homologous immune rabbit sera. Table I compares the activity of a *Leptospira canicola* antigen prepared by NaOH extraction with that of a standard suspension. The former, although fixing more complement with a 1:20 dilution of immune rabbit serum, gave a maximal serum titer of only $\frac{1}{16}$ that of the suspension. Likewise, in the tests with patients' sera the NaOH antigen proved to be less sensitive than the suspension.

TABLE I. COMPLEMENT FIXING ACTIVITY OF *L. Canicola* ANTIGENS

DILUTION OF SERUM	UNITS OF COMPLEMENT FIXED					
	RABBIT IMMUNE SERUM		SERUM OF PATIENT O. I.		SERUM OF PATIENT R. K.	
	SUSPENSION	NaOH EXTRACT	SUSPENSION	NaOH EXTRACT	SUSPENSION	NaOH EXTRACT
1:10	–	–	3.0	3.0	3.4	0
1:20	11.0	13.5	3.0	0.5	3.4	0
1:40	10.5	5.5	3.0	0	3.4	0
1:80	10.5	0.7	2.0	0	3.4	0
1:160	7.5	0	1.2	0	3.4	0
1:320	6.0	0	0.5	0	2.4	0
1:640	2.9	0	0	0	1.6	0
1:1280	0.7	0	0	0	0	0
1:2560	0	0	–	–	–	–

Tests With Immune Animal Sera.—Using the standard dose of antigen in a "line test"[9] it is possible to study the antibody content of a serum either by testing a single concentration of serum against varying amounts of complement or by testing dilutions of serum against a fixed amount of complement. The serum of a rabbit immunized with *Leptospira pomona* and the serum of a

TABLE II. COMPLEMENT FIXATION AND AGGLUTINATION BY RABBIT AND GUINEA PIG IMMUNE SERA WITH HOMOLOGOUS AND HETEROLOGOUS TYPES OF LEPTOSPIRAE

TYPE*	RABBIT IMMUNIZED WITH *L. pomona*			GUINEA PIG IMMUNIZED WITH *L. canicola*		
	UNITS OF COMPLEMENT FIXED WITH SERUM 1:10	SERUM TITER WITH 2 UNITS COMPLEMENT	AGGLUTININ TITER	UNITS OF COMPLEMENT FIXED WITH SERUM 1:10	SERUM TITER WITH 2 UNITS COMPLEMENT	AGGLUTININ TITER
1	5.3	320	<80	2.0	20	<100
2	4.8	160	<80	7.1	80	400
3	25.0	2,560	81,920	0.9	10	<100
4	3.3	40	<80	0.9	10	400
5	7.2	320	<80	1.8	20	<100
6	22.0	1,280	2,560	1.6	20	<100
7	8.8	640	640	0.5	10	<100
8	7.6	80	<80	1.5	40	200
9	7.6	80	5,120	1.5	20	400

*1. *Leptospira icterohemorrhagiae;* 2, *L. canicola;* 3, *L. pomona;* 4, *L. grippotyphosa;* 5, *L. bataviae;* 6, *L. autumnalis* AB (Akiyama A); 7, *L. autumnalis* A (Rachmat); 8, *L. hebdomadis* (NIH); 9, *L. hebdomadis* (Beeson).

guinea pig immunized with *L. canicola* were tested by both methods and the results compared with agglutination tests using live cultures (Table II). By complement fixation each serum reacted with suspensions of all the types tested, although the most marked reaction was obtained with the homologous type. In general, the higher the titer of the serum using two units of complement, the greater the amount of complement fixed by a 1:10 dilution of serum. The test using varying amounts of complement had the advantage of not requiring preliminary titration of complement[6, 9] and made it unnecessary to allow for the unequal anticomplementary effects of various antigens.

Tests With Sera From Patients.—Table III shows the amount of complement fixed by 1:5 dilutions of the sera of three patients with leptospirosis. The agglutination test indicated that Patient G. B. was infected with *L. grippotyphosa.*[14] A suspension prepared from this type also fixed more complement than antigens prepared from other types. In the case of Patient O. I., however, the serum was almost equally reactive with five types by complement fixation although agglutination was obtained only with *L. canicola.* With the serum of Patient R. K., the complement fixation reaction was more type-specific. Serum specimens from ninety patients that were negative for leptospirosis by agglutination fixed insignificant amounts of complement (less than 0.5 unit) when tested in a 1:5 dilution against suspensions of the nine leptospiral types.

TABLE III. COMPLEMENT FIXATION AND AGGLUTINATION BY PATIENTS' SERA WITH HOMOLOGOUS AND HETEROLOGOUS TYPES OF LEPTOSPIRAE

	PATIENT G. B.		PATIENT O. I.		PATIENT R. K.	
TYPE*	UNITS OF COMPLEMENT FIXED WITH SERUM 1:5	AGGLUTININ TITER	UNITS OF COMPLEMENT FIXED WITH SERUM 1:5	AGGLUTININ TITER	UNITS OF COMPLEMENT FIXED WITH SERUM 1:5	AGGLUTININ TITER
1	1.3	<100	4.4	<100	2.1	200
2	0.6	<100	4.4	3,200	4.1	3,200
3	1.5	<100	4.4	<100	1.3	<100
4	5.6	3,200	2.5	<100	1.5	<100
5	1.5	<100	2.5	<100	1.9	<100
6	1.5	<100	2.2	<100	1.5	<100
7	0.6	<100	1.6	<100	0.9	200
8	1.5	<100	4.2	<100	1.6	<100
9	0.5	<100	3.9	<100	1.0	<100

*See footnote to Table II.

DISCUSSION

In addition to requiring a relatively small amount of antigen and the economy in other materials and glassware, one of the greatest advantages of the plate complement fixation test is the facility with which one may investigate the quantitative relationships of antigen, antibody, and complement. Thus, it appeared that increasing the concentration of antigen beyond that used would not increase the sensitivity of the complement fixation test. On the other hand, the plate test should be more sensitive than the conventional

tube test in which the fixation of less than 2 units of complement is usually not detected. Much more extensive use of the plate test with the serum of patients will be required to determine its usefulness as a diagnostic procedure.

SUMMARY

The plate complement fixation test previously used with viral antigens has been applied to the detection of leptospiral antibodies in animal and human sera. The relatively small amounts of antigen required and the simplicity of the procedure suggest that it will be a useful method of studying leptospiral complement fixation.

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A SMALL PERFUSION APPARATUS FOR THE STUDY OF SURVIVING, ISOLATED ORGANS

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SINCE the report on a glass perfusion apparatus for small organs by one of us (J. A. Long) in 1947,[1] an entirely new plastic model has been developed. Two of the important features of the earlier model have been retained: (1) the perfusion may be done with small amounts of blood; (2) the organ being perfused may be kept under continuous microscopic observation, and in position for photography. The availability of a good thermostable plastic* has permitted the replacement of glass to the point where only two glass parts are now used—the top of the chamber in which the organ is placed, and a glass pipette. These may also be made of plastic; but in the first case the glass has been retained for better microscopic observation; and in the second, it is a matter of convenience, since standardized glass pipettes are more easily available. Other recent developments that have proved valuable to the perfusionist are: (1) the nontoxic silicones used to create nonblood-wetting surfaces on glass, metal, cloth, and plastic parts; and (2) plastic tubing.

The use of plastic tubing has permitted a great deal of latitude in the designing of the new apparatus. And so in contrast to the compact glass machine that was fragile and somewhat troublesome to assemble, an apparatus has been built with separable, durable, but easily replaceable parts made in standard molds and joined together for a variety of functions by plastic tubing.

The apparatus has been used for small organs, the largest having been the liver of a 1-month-old rat. For larger organs it would only be necessary to design an organ chamber larger than the one described. A variety of such chambers may be built and adapted to the rest of the apparatus, depending upon the size and shape of the organ to be studied.

Figs. 1 to 3 are photographs of the apparatus taken from different angles. Fig. 3 shows the complete assembly of one machine. Ordinarily a second machine is joined to this by a long connecting rod—in order that a second organ may be studied simultaneously under the conditions of controlled variables. The motor shown may be used to motivate two separate pumps. Other parts not shown in this assembly are: (1) the constant temperature water bath with pump for circulating water to maintain a desirable temperature in the organ; (2) the bottles and siphons suspended by adjustable pulleys at the appropriate height above the dialyser, to the bottom chamber of which a fluid of a desired composition runs, giving certain substances to, and taking others from the blood in partial imitation of a kidney (see Fig. 41); (3) a side-arm that may be used as a continuous feeding device for the addition of nutrients or other substances at a constant rate throughout the experiment (see Fig. 42); (4) the 95 per cent O_2 and 5 per cent CO_2 tank with valves, filter, and bubbler bottles.

A complete set of detailed drawings of the separate parts of the apparatus is also presented. The photographs and drawings are labeled as completely as possible with some description of the various assemblies in the legends. In order to elaborate further on the

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*A polyester (Selectron) purchased from the Pittsburgh Plate Glass Company, Pittsburgh, Pa.

†Deceased June 17, 1953.

apparatus, and particularly to explain the use of its many parts, it has seemed desirable to describe all of the steps taken in assembling the apparatus and the technical details of a typical experiment.

Fig. 2*B* is a schematic diagram of the main parts of the apparatus drawn to indicate the directions of flow of the various fluids used.

Assembling the Apparatus.—The apparatus is usually assembled on the day before the planned experiment. This requires about two hours of one person's time. Before the parts are put together they are wiped scrupulously with lintless cloth or preferably sheets of silicone paper; and with the aid of a magnifier, brushed clean of any material that would prevent good seals. All parts may be treated with a spray of thinned silicone,* excess of which is wiped off with silicone paper. The separate parts are then heated in an oven at 100° C. in the presence of evaporating NH_3 for fifteen minutes. Apparently after a few of such treatments, a layer of polymerized silicone remains on the treated surfaces and further applications may be unnecessary. After applying the silicone to the steel tubing, the channels in the plastic and the short length of rubber tubing sometimes used in preference to plastic between the pump valves, it is thoroughly blown out with a blast of filtered air. Plastic tubing need not be silicone-treated, but is washed thoroughly by siphoning distilled water through it. The plastic tubing is cut into standard lengths for the various connections and stored as such. It is adapted to the steel tubing in the plastic parts in such a way that after autoclaving it may be pushed further up and more tightly onto the steel tubing. The plastic parts that turn, one on the other (e.g., stopcocks), are greased lightly with a non-toxic silicone lubricant.† The skill of having enough and yet avoiding excess can be acquired only with practice. On some occasions it may be necessary to take the greased stopcocks apart and remove the excess of grease from the channels. Grease should be applied in minimal amounts, even though it is expected that excesses of it thrown into the circulation would be stopped in the filter. Two thin lines applied with a syringe and needle above and below the channels usually suffice, but an alternative method is to spread grease over the upper part uniformly with the fingers and wipe its channels free before engaging it to the stopcock base. Grease is used on the two blood distributing stopcocks No. 14 and No. 21, the small air trap (Fig. 23), the stopcock of mercury manometer No. 16, the pressure equilibrator, the filter, and for sealing the glass top to the organ chamber. A thin line of grease in the form of a circle is delivered onto the top of the organ chamber, and onto both of the palstic discs of the filter by ejecting it from a loaded tuberculin syringe through an 18-gauge needle.

Sterilizing the Apparatus.—When all of the parts are assembled, as shown in Figs. 1 to 3, in the insets provided on the small base plate No. 57, the apparatus is autoclaved at 15 pounds pressure for one-half hour. The small plate when detached from the larger base carries all of the plastic parts except the filter and dialyser. These are connected as shown and autoclaved also. When the apparatus has been sterilized and allowed to cool, some of its parts require a final tightening, especially the tops to the pump valves and the wing nuts on the filter and dialysing clamps. The upper part of the pressure regulator (Fig. 20) is not autoclaved. The lower part (Fig. 21) with the rubber dam and washers in position are autoclaved; and later, the upper part is screwed tightly into position.

The filter and bubbler bottles for the $O_2 - CO_2$ mixture may be autoclaved with the rest of the apparatus, as may the nutrient feeding tube. The tubing to and from the solution against which the blood is to be dialysed is not sterilized, since the cellophane acts as a bacterial filter. Small venous and arterial cannulae made from stainless steel needles of different gauges are adapted to steel taps by plastic tubing as shown in Fig. 9. Two ligatures of silk thread are placed loosely in the organ chamber for later use. Should there be any objections to autoclaving the apparatus, a sterilizing solution may be pumped through

*Dow-Corning's D. C. 1107 diluted with petroleum ether, 1:10 or more.
†Dow-Corning's high-vacuum heavy silicone grease.

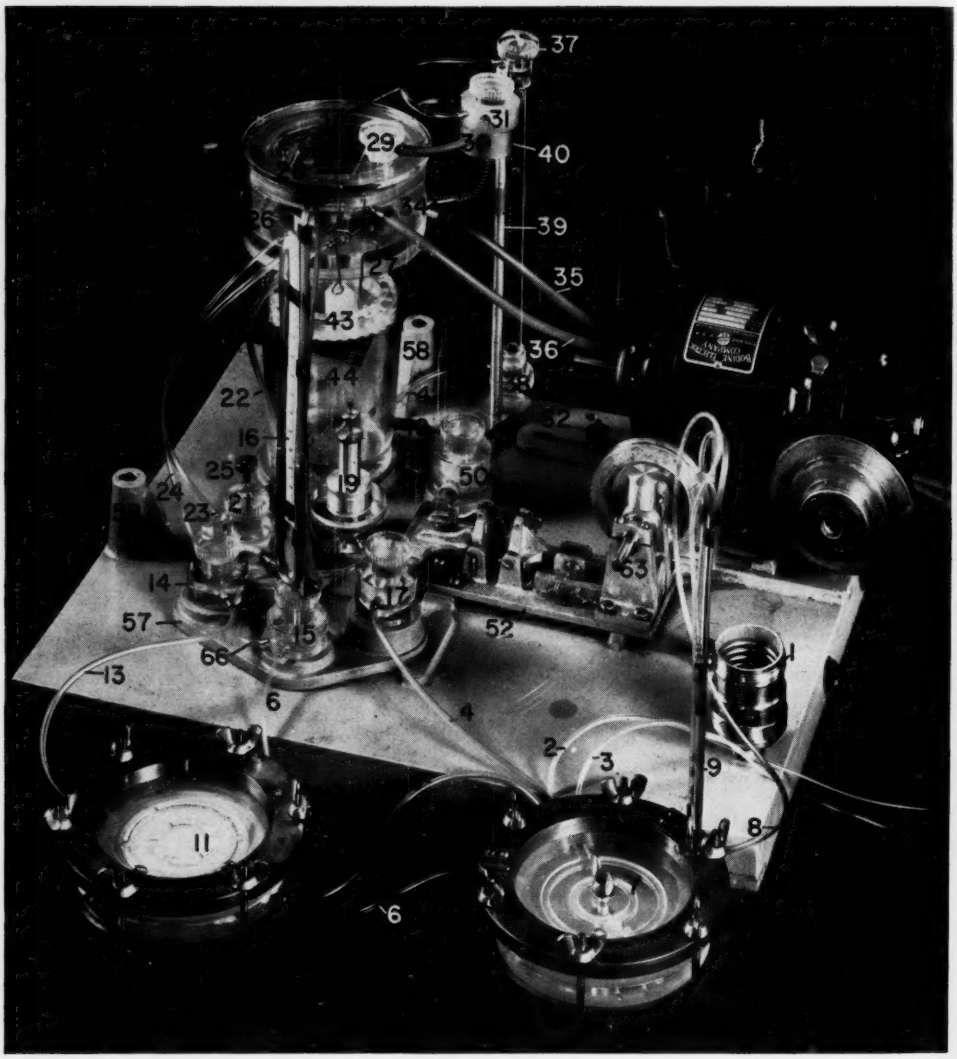


Fig. 1.

Figs. 1, 2*A*, and 3.—*1*, Light bulb socket (resistance). *2*, Tube to dialyser from Tyrode's bottle. *3*, Tube from dialyser to drain. *4*, Tube from pump valve to dialyser. *5*, Tube from top section of dialyser to filter. *6*, Tube from top section of dialyser to manometer. *7*. Dialyser (Fig. 36). *8*, Tube from bottom section of dialyser to manometer No. 9. *9*, Mercury manometer. *10*, Cellophane sheet between plastic discs of dialyser. *11*, Filter (Fig. 38). *12*, Fine mesh cloth filter between plastic discs. *13*, Tube from filter to stopcock No. *14*. *14*, Blood distributing stopcock (Figs. 23-27). *15*, Stopcock (Figs. 34, 35) for manometer No. *16*. *16*, Mercury manometer (Fig. 33). *17*, Outlet valve (Figs. 17 to 19). *18*, Tube from stopcock No. *14* to pressure regulator. *19*, Pressure regulator (Fig. 20). *20*, Tube from pressure regulator to stopcock No. *21*. *21*, Blood distributing stopcock. *22*, Tube from stopcock No. *21* to by-pass to reservoir. *23*, Tube from stopcock No. *21* to organ chamber. *24*, Tube from stopcock No. *14* to arterial cannula. *25*, Centering pin for adapting small base plate to large one. *26*, Water jacket (Fig. 10). *27*, Wire spring fastener (Fig. 6). *28*, Organ chamber (Fig. 8). *29*, Plug in opening in glass cover to organ chamber (Fig. 7). *30*, Tube from organ chamber to pressure equilibrator. *31*, Pressure equilibrator (Figs. 28 to 31). *32*, Tube from equilibrator to reservoir (air). *33*, Tube from equilibrator to flow meter. *34*, Tube from equilibrator to reservoir, with spiral wire stiffener. *35*, Water inlet tube to warm water jacket. *36*, Water outlet tube from warm water jacket. *37*, Top of flow meter (Fig. 32). *38*, Bottom of flow meter. *39*, Steel rod for suspending pressure equilibrator. *40*, Spring steel band for holding equilibrator. *41*, Swivel-joint holder for steel rod No. *39*. *42*, Tube from bottom of flow meter to reservoir. *43*, Blood spreader (Fig. 13). *44*, Blood reservoir and aerating chamber (Fig. 12). *45*, Posts for adapting plastic tubing holding cannulae (Fig. 9), *46*, Blood inlet to organ chamber (*A*, Fig. 9). *47*, Gas inlet to reservoir (*B*, Fig. 12). *48*, Blood outlet from reservoir to valve No. *50*. *49*, Tube from reservoir to pump valve No. *50*. *50*, Inlet valve (Figs. 14 to 16). *51*, Rubber tube between valves. *52*, Pump assembly (Fig. 4). *53*, Three-unit pulley on pump. *54*, Three-unit pulley on motor. *55*, Constant speed motor. *56*, Aluminum base plate. *57*, Small aluminum base plate for insetting plastic parts. *58*, Slots for legs of microscope table and arm rest. *59*, Adjustable leg for microscope table. *60*, Microscope table. *61*, Binocular microscope on movable wooden base. *62*, Slot for adjusting distance of motor from pump. *63*, Shaft for connecting twin apparatus. *64*, *Electric* switch for motor. *65*, Electric switch for lamp resistance No. *1*. *66*, Tube from stopcock *14* to manometer base No. *15*.

all of the parts of the apparatus, and then washed out with saline solution. Selectron, the plastic used in fabricating all of the parts of the apparatus and Transflex* plastic tubing may be autoclaved without trouble.

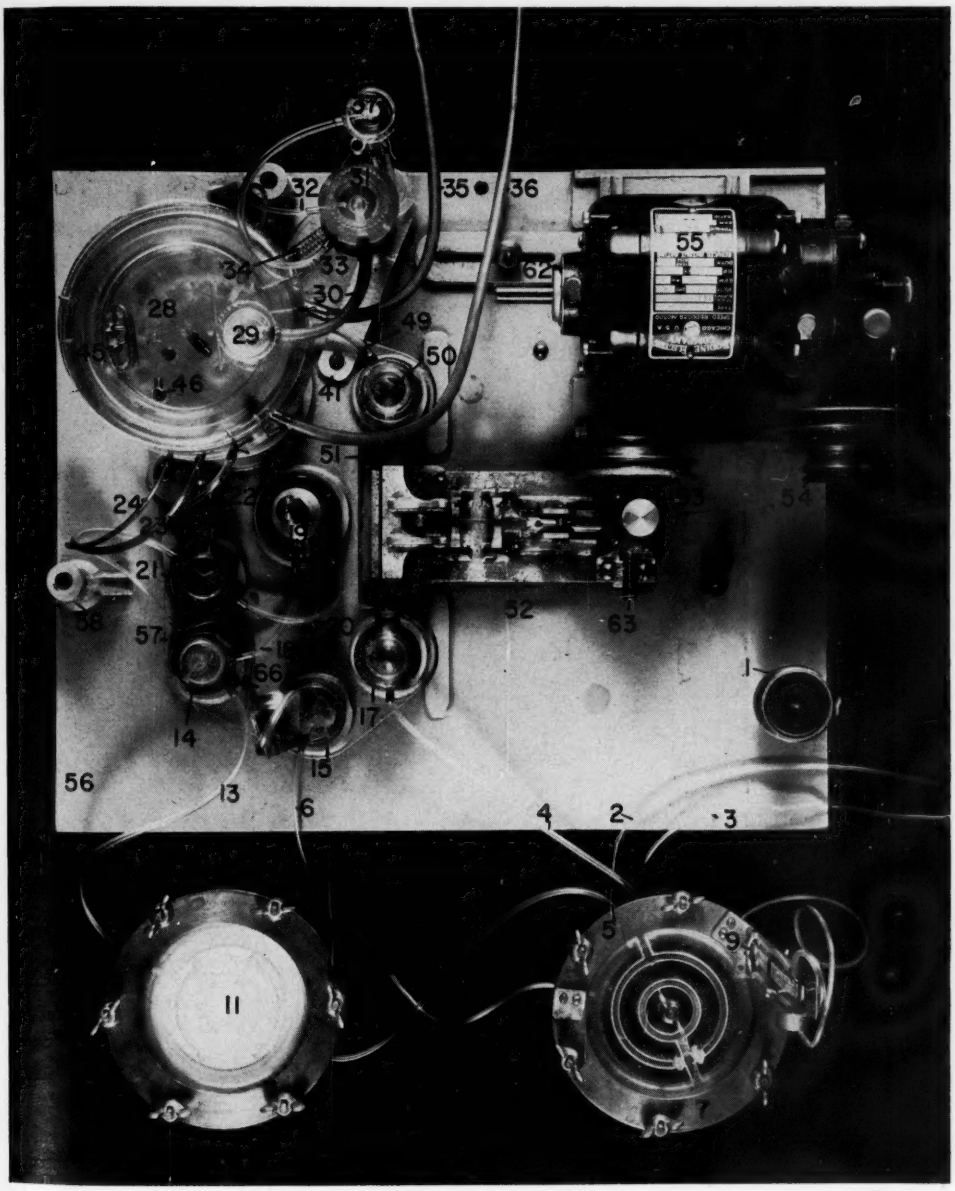


Fig. 2*A*. (*For legend see Fig. 1.*)

Testing the Apparatus.—After sterilization, the apparatus is tested for leaks with sterile White's fluid.[2] This is poured into the reservoir (about 80 ml.) and the main pump motor is started after engaging the rubber tubing No. 51 (Fig. 2*A*) between the compressing jaws. Air clearance is facilitated by holding the dialyser and filter in a position that

*Procured from the Irvington Varnish and Insulator Company, Irvington, N. J.

permits the air to rise and to be ejected from the exit tubes by the fluid. By removing tube *H* of manometer (Fig. 33) at joint *G*, momentarily, and having the stopcock turned so as to open the correct channel, White's fluid may be left between the blood channels and the mercury. During the testing of the apparatus, the pressure regulator No. 19 is adjusted in such a way that most of the fluid returns to the reservoir via tube No. 22 to the by-pass, leaving only a small stream under low pressure going through tubing No. 24 to the arterial cannula.

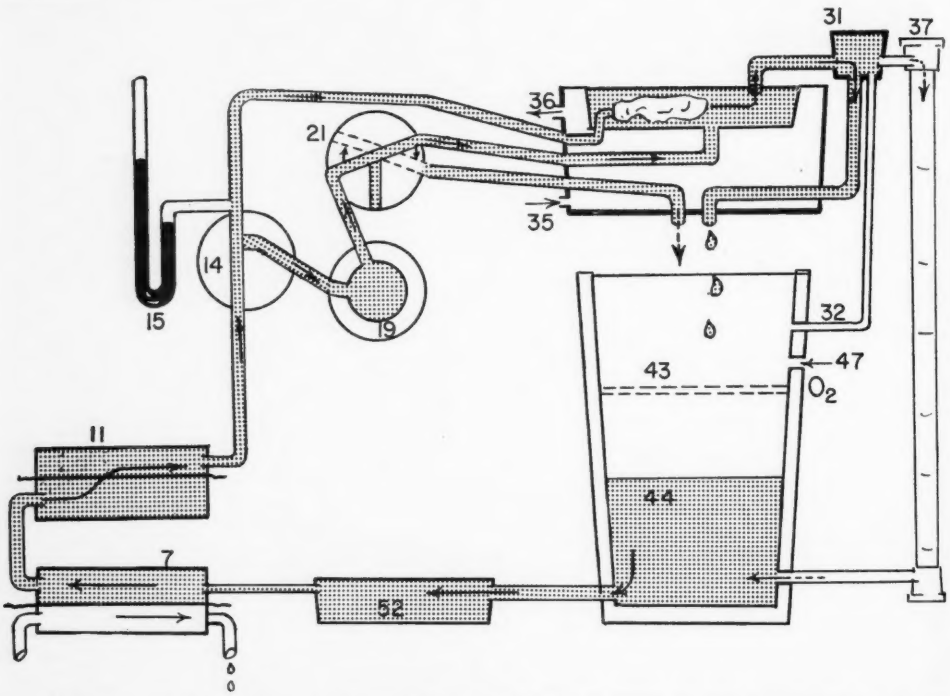


Fig. 2*B*.—A diagrammatic representation of the main parts of the apparatus shown in Fig. 2*A*, illustrating the pathways and direction of flow of the various fluids used in a typical experiment. The "open" system of perfusion is shown. In a "closed" perfusion experiment, the main vein of the organ is also cannulated; the blood from the organ does not flow into the organ chamber, but passes through plastic tubing to return to the reservoir via parts No. *31* and No. *34*. Nor does the blood flow into the organ chamber via tube No. *23*, but by-passes through tube No. *22*. The numbers in this diagram correspond to those in Fig. 2*A*. In cases of optional flow routes from the distributing stopcocks the channels are shaded differently.

The heating unit having been previously adjusted for a given temperature in the water jacket and organ chamber, the water pump is turned on and the water tightness of the jacket tested. It is necessary to keep the warm water supply well below the level of the water jacket in order to avoid undue back pressure and leakage. It may be desirable to attempt some adjustment of the type of stream to be propelled through the arterial cannula during the period of testing. By observing the type of flow or jet emerging from the arterial cannula, as adjustments are made on the pressure regulator, and at points *6* and *11* on the pump, any variety of circulation from a continuous flow with minimal pulse pressure to a strong jet with high pulse pressure may be obtained. Since the end result would be determined by the type of organ cannulated and particularly by the resistance offered by its vascular channels, these adjustments have relatively little value until one has made trial runs with any given organ. They are only considered to be approximate adjustments; and are also safety precautions against ruining a preparation by rupturing blood vessels and causing interstitial hemorrhage in the first few seconds of the perfusion.

Preparation of the Blood and Nutrients for Perfusion.—Five rats are bled by cardiopuncture, each contributing about 7 ml. They may be bled again after two to three weeks.

The blood is defibrinated by shaking gently in a flask containing glass beads. It is filtered through a previously dampened sterile glass filter, and the following substances are added: 5 mg. of sodium heparin, 8 mg. of potassium penicillin G, and 20 mg. of dihydrostreptomycin sulfate. Before the organ is perfused the blood may be put into the apparatus and circulated for approximately two hours for further filtration, and if desired, for dialysis of pressor or so-called "toxic" substances. The blood is added to the White's fluid already in the apparatus in prearranged proportion. A ratio of 1:4 of blood to White's fluid has been satisfactory in studies on the liver. Red cells washed several times in isotonic saline solution and resuspended in White's fluid may be desirable in some experiments.

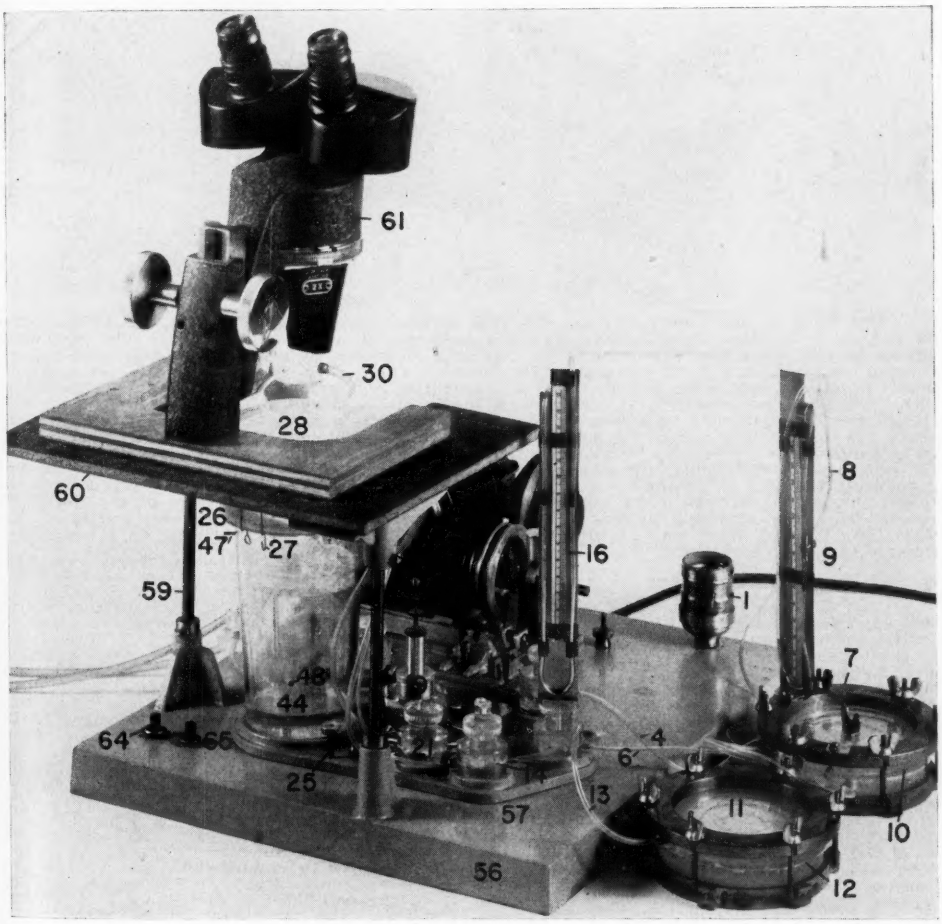


Fig. 3. (*For legend see Fig. 1.*)

Preparation of the Organ for Perfusion.—With the blood well mixed with White's fluid or any other desirable nutrient, and well filtered, with the temperature regulator functioning properly, with the 95 per cent O_2 and 5 per cent CO_2 mixture flowing into the blood reservoir at a rate previously determined to be adequate, with no evidence of leakage around stopcocks or other parts, with the blood being dialysed against a fluid of prearranged constitution, with White's fluid or other nutrients siphoning slowly into the reservoir at a rate calculated to compensate for certain losses by dialysis, and with the pressure and pulse regulated approximately as intended for the experiment, an organ is removed from a heparinized donor. The

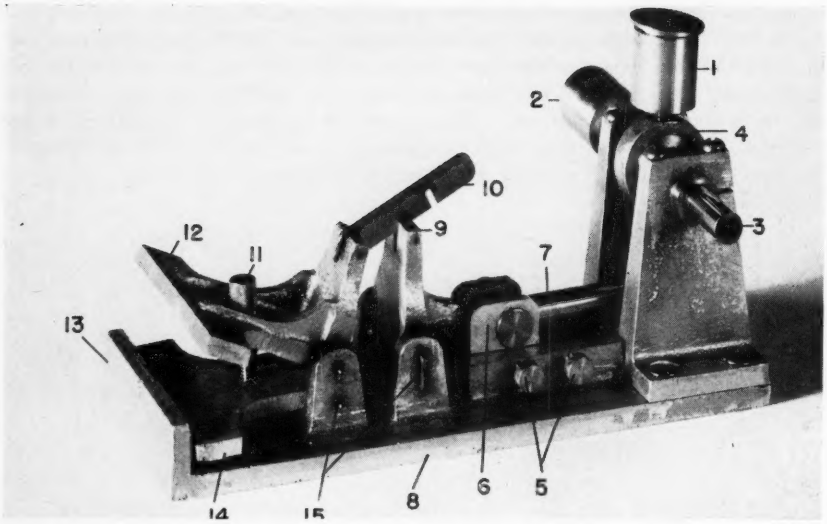
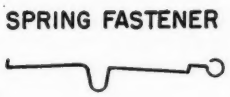
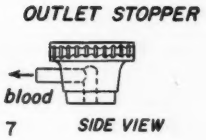
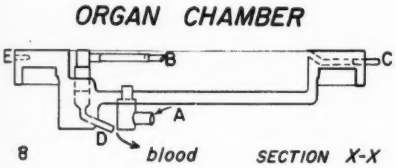


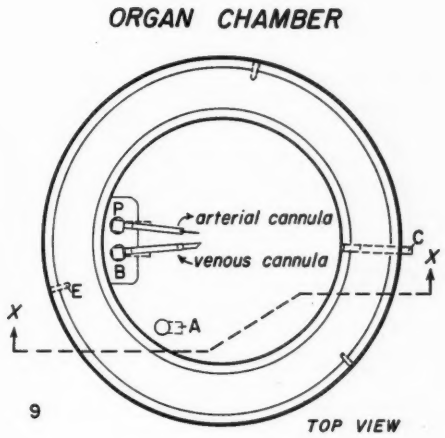
Fig. 4.—The pump assembly may be seen in four views (Figs. 1 to 4). It is connected at (*2*) to a constant speed motor, and the beat-rate is varied by the two sets of pulleys shown in Fig. 2*A*. Shaft (*3*) turns in two sets of bronze brushings and a bronze journal bearing running in a thick lubricant from oil cup (*1*). This shaft also motivates a second pump to a twin apparatus by means of a connecting rod and a flexible coupling attached at (*3*). Through the eccentric at (*4*) and a shank and a knuckle joint a rocking motion is transmitted to shaft (7) and then to rocker (*9*). Fulcrum axle (*6*) may be adjusted along shaft (7) for various stroke lengths by set screws (*5*). Rocker (*9*) moves back and forth on a simple axis (steel pin); and when the notch on connecting piece (*10*) is engaged in it, the movement is transmitted to upper jaw or compressor (*12*). A rubber tube shown in Fig. 2 connects the two valves and carries the blood from one to the other, when compressed between the jaws (*12*) and (*14*). The lower jaw (*14*) is adjustable on base plate (*8*) by the screw (*11*). By adjusting pulleys No. *34* and No. *35* (Fig. 2*A*) fulcrum (*6*) and lower jaw (*14*), any type of pulse may be imitated or produced. A lip (*13*) extending from the aluminum base plate (*8*) retains the rubber tube during compression.

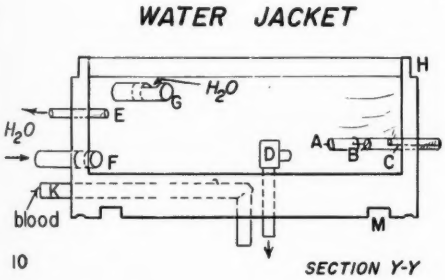
Fig. 5, Glass top to organ chamber No. *28* Fig. 2*A*. Fig. 6, Spring wire fastener in two lengths (No. *27*, Fig. 1). Three long ones fit under the water jacket and over the glass top in the ground depressions. Three short ones fit under the water jacket and into slots drilled in the organ chamber (see *E*, Figs. 8 and 9). Fig. 7, Stopper for blood outlet, No. *29*, Fig. 1. Fig. 8, Cross section of organ chamber, No. *28*, Fig. 1. The outer lip is ground and tapered to fit over water jacket (Fig. 10). Blood enters organ chamber through steel elbow *A* after having circled through the warm water jacket in plastic tubing. If the main vein is cannulated, it is attached at *B;* and the blood passes out through a short length of plastic tubing in the water jacket between *D* of this figure and *E* of Fig. 10. At *C* a 25 cm. length of plastic tubing is attached and suspended at a desirable level. This is a multipurpose tube, being used for expansion of chamber fluid, for altering the pressure around the organ intermittently, etc. Channels for wire fasteners are shown at *E*. Fig. 9, Top view of organ chamber. Labeled as in Fig. 8. Fig. 10, Cross section of warm water jacket, No. *26*, Fig. 1. *A*, *B*, *C* = stainless steel tubes to which plastic tubing circling in the water jacket is attached. *A* joins *D* of Fig. 10 = by-pass; *B* joins *A* of Fig 8; *C* joins base of *P* of Fig. 9. *E* is joined by plastic tubing to *D* of Fig. 8 = base of venous cannula. Water warmed by a thermostatic regulator is pumped into water jacket at *F* and leaves at the exit *G*. The organ chamber fits snugly over the tapered rim of the water jacket *H*. The top of the reservoir (Fig. 12) fits loosely into the groove at *M*. Blood returns to the reservoir from the equilibrator, No. *31*, Fig. 1 through *K* and drops on spreader (Fig. 13). Fig. 11, Top view of water jacket. Labeled as in Fig. 10. Fig. 12, Side view of reservoir with blood spreader loosely fitted across it about ⅓ of the distance down from the top. O_2 after passing through a "bubbler" bottle and a Berkefeld candle enters top of reservoir at *B*. A plastic tube connects steel tube *A* with steel tube *A* of equilibrator (Figs. 30 and 31); and when it is left open, air from the reservoir travels up it, and returns with the blood from the equilibrator via *D*, Fig. 30. Blood leaves the bottom of the reservoir via *C* to tube *B* of inlet valve (Fig. 15). When the flow gauge has been used the blood is returned to the reservoir through *D*. The tube of a 26 gauge stainless steel hypodermic needle with the pointed end outside is engaged in a channel in the plastic, and used for adapting the plastic capillary tubing from the feeding device shown in Fig. 42. When this is not in use a short length of polyethylene tubing is left adapted to the needle and heat-sealed at the opposite end. Fig. 13, Blood spreader. Blood dropping onto this plastic disc runs through some or all of the horizontal channels and down the side of the reservoir. The vertical channels permit equilibration of pressure.

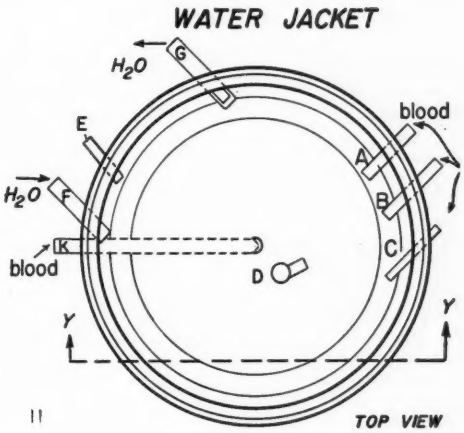


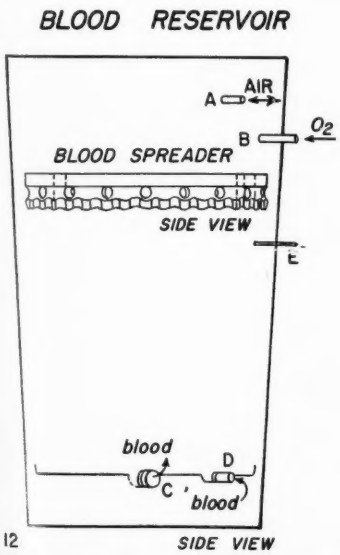


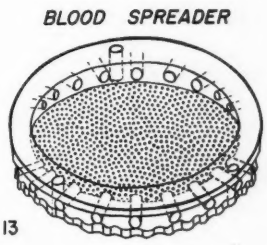






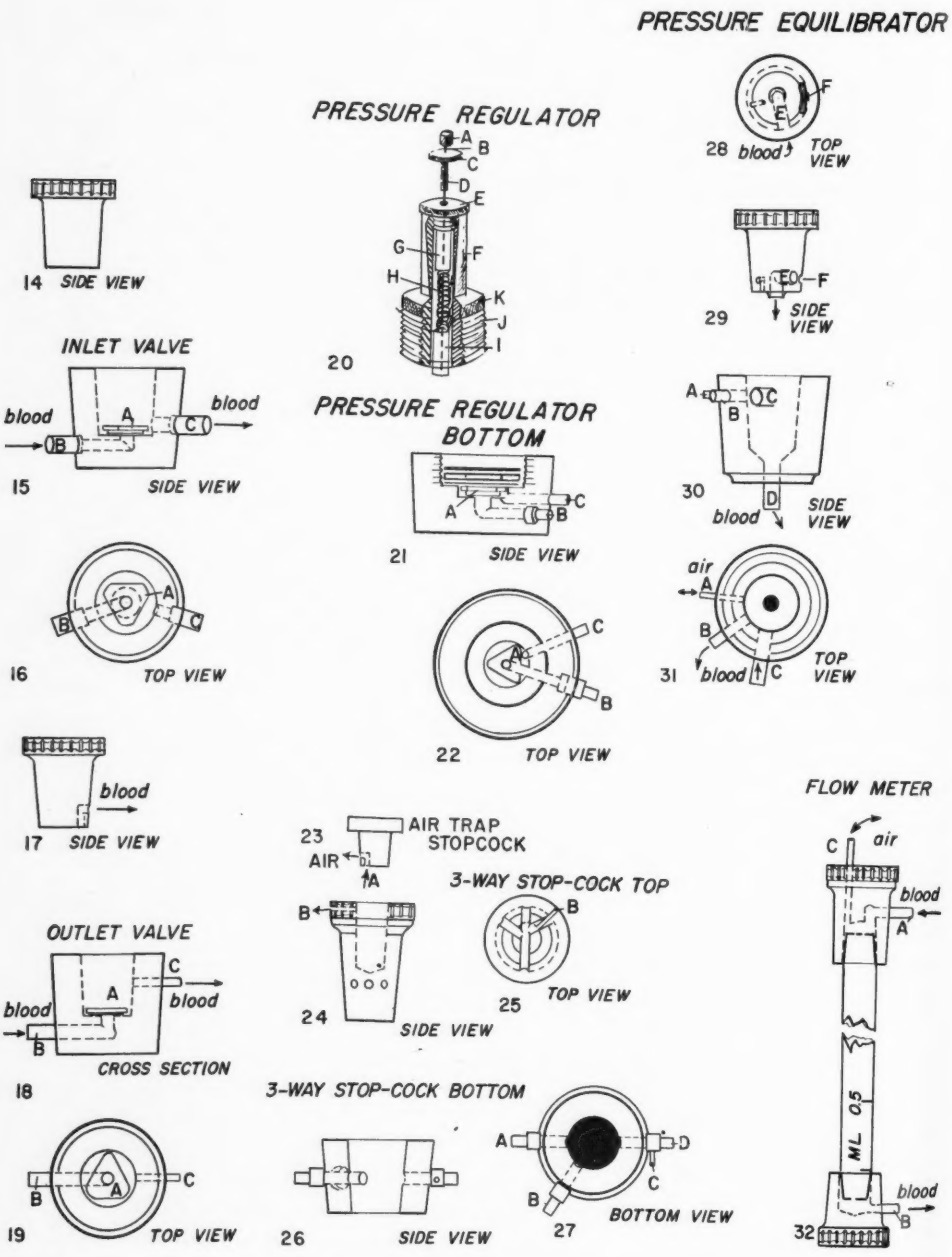






Figs. 5-13. (All drawings were made to scale and reduced ½.) (*For legends see opposite page.*)

glass cover is removed from the organ chamber which is partly filled with White's fluid and the organ cannulated while immersed. A binocular microscope and a bright spotlight are usually necessary during the operation of drawing the artery onto the cannula with the help of two fine watchmaker's forceps. A drop of 1 per cent papaverine proves useful in relaxing the artery if it is in spasm.



Figs. 14-32. *(For legends see opposite page.)*

At the time the blood is added to the apparatus, the circulation is directed entirely over the by-pass leaving the arterial tubing and cannula filled with White's fluid and submerged below the level of the White's fluid partly filling the organ chamber. *Air must not get into the cannula.* When the organ is attached to the arterial cannula using a silk ligature, a test is made by manual manipulation of the pump and observance of the outflow of dark blood from the vein. This blood is washed out of the organ by the White's fluid remaining in the arterial tubing.

"Open" Perfusion.—In the perfusion of some organs or isolated tissues, it may be unnecessary or impracticable to cannulate the main artery and vein and otherwise secure complete hemostasis so that the entire outflow from the organ is carried into a single channel, leaving the chamber fluid clear of blood. In such cases only a single vessel—usually the main artery—need be cannulated and the effluent blood is allowed to flow freely into the organ chamber through one or more veins or other blood vessels, and to mix with the well-oxygenated blood diverted from the by-pass and made to flow into the organ chamber through tube *A*, Fig. 8.

The glass top is then applied to the organ chamber using a silicone grease seal and plugging in the plastic stopper No. 29 which is used as the only exit in experiments in which the main vein is not cannulated. In experiments performed on the liver, the portal vein is attached to the arterial cannula and the blood leaving the liver enters the organ chamber via the hepatic veins or the vena cava usually left in continuity with the hepatic veins. In such a case, it seems that a highly desirable backflow takes place through the hepatic artery keeping the tissues supplied by that vessel adequately nourished and oxygenated. Since the perfused blood flows freely from the liver into the organ chamber the cannula usually used for venous outflow is available for collecting bile. This technique is described in a separate report.[3]

"Closed" Perfusion.—When an organ is perfused by cannulating the main artery and vein and attaining complete hemostasis elsewhere in the tissue by ligatures and thermocoagulation, a solid plug is substituted for the one shown as No. 29, Fig. 1, functioning as

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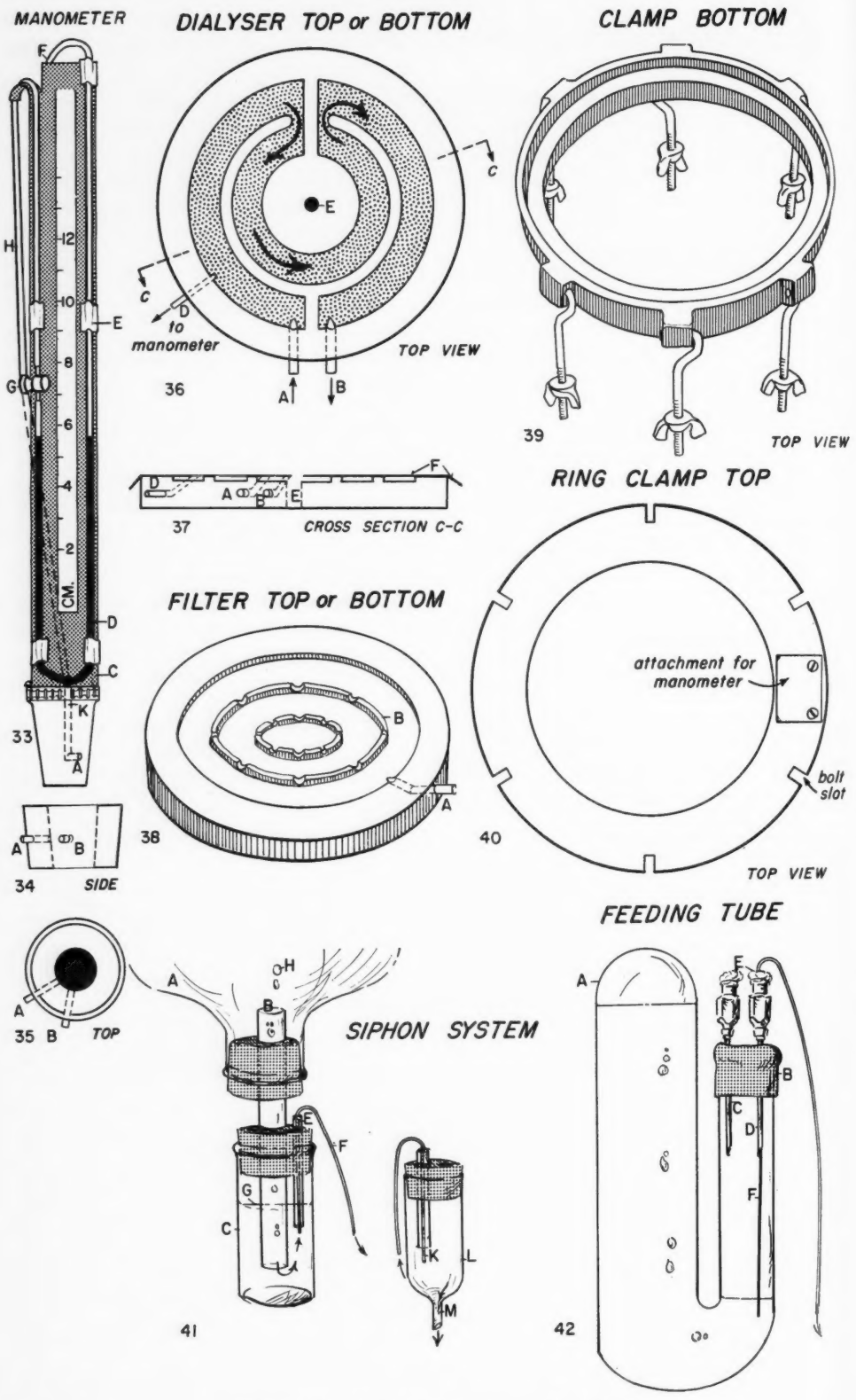
Fig. 14, Stopper for inlet valve chamber, No. *50*, Fig. 1. Fig. 15, Inlet valve chamber, side view. Fig. 16, Inlet valve chamber, top view. Valve *A* is made of glass, stainless steel, or plastic. Blood from reservoir enters at *B* and leaves at *C*. Figs. 17-19. Outlet valve parts. No. *17*, Fig. 1. Steel tube *B* is connected with steel tube *C*, Fig. 15 of inlet valve by rubber or plastic tubing compressed rhythmically by the pump. Steel tube *C* is connected by plastic tubing to the dialyser. Fig. 20, Pressure regulator, No. *19*, Fig. 1. Assembly of upper part: *A* = knurled brass top to steel pin *B* which runs through to lower aluminum cylinder *I*. This pin moves *I* up or down on the valve, the level and pressure being regulated by the threaded adjustable brass parts (*C*, *D*, *E*) and the steel spiral spring *H* which may be compressed within aluminum cylinders *G* and *I*. Parts *C* and *D* are joined and slide freely on *B*. *D* threads into *E* and adjusts the tension on spring *H*. *E* threads into aluminum casing *F* and is kept tight. Casing *F* and threaded base *J* are milled from the same block of aluminum. The base has a knurled shoulder *K* to facilitate turning and tightening in the plastic base (Fig. 21), Figs. 21 and 22, Plastic bottom of pressure regulator. *A*, instead of plastic, glass or stainless steel valve; *B* = inlet from blood-distributing stopcock No. *14* (Fig. 2*A*); *C* = outlet to distributing stopcock No. *21* (Fig. 2*A*). Above the valve in Fig. 21 in ascending order are: a thin round sheet of rubber dam; a rubber washer; and a stainless steel gasket. Figs. 23-27, Parts of the blood-distributing stopcock No. *14* (Fig. 1). Fig. 23 shows the secondary stopcock that turns in the upper part of the main stopcock (Fig. 24) and permits it to be used as an air trap. Blood forces trapped air into *A* and out through *B*, Fig. 24. The bottom of the main stopcock, Figs. 26, 27, is open. Stainless steel tubes for adaption of plastic tubing are shown at *A*, *B*, and *D*. A smaller gauge tubing leading to the arterial cannula is adapted to *C*, a side arm of *D*. *A* is connected to the filter No. *11* (Fig. 1); *B* to the base of pressure regulator No. *19*; *D*, to the manometer base No. *15*. The details of stopcock No. *21* (Fig. 2*A*) are the same as No. *14* except for the air trap and the small side arm *C* in tube *D*, which are absent. Figs. 28-31, Parts of the pressure equilibrator No. *31*, Fig. 1. This is a cup-trap with a three-way stopcock. Steel tube *A* is connected with the blood reservoir by a plastic tube and this allows air (mostly O_2) to enter the cup and continuously move out with the blood through the exit *D* to re-enter the reservoir via the plastic tube No. *34*, Fig. 1. The blood enters the cup from the organ chamber through *C* and tube No. *30*, Fig. 1; and is kept at the level of the blood in the organ chamber, leaving an air space above it. For estimating blood flow rate, the stopcock is turned to the left until channel *E*, Fig. 29, is in line with exit *A*, Fig. 31 in the cup. Lateral groove *F*, Figs. 28, 29, then carries the blood from *C* to exit *B*, Figs. 30, 31. This blood runs into the measuring pipette of the flow meter. Fig. 32, The flow meter or gauge is a calibrated glass pipette ground to fit into a plastic top and bottom as shown. Blood enters the top at *A* from the equilibrator and leaves the bottom at *B* to return to the reservoir via plastic tubing. During the measurement of the flow-rate the plastic tube carrying the blood to the reservoir is clamped off and the filling of the pipette to certain levels is timed with a stop watch. An opening to the air, *C*, plugged with cotton, keeps the pressure equilibrated in the flow meter.



Figs. 33-42. *(For legends see opposite page.)*

a blood exit. The venous cannula is connected by plastic tubing to exit *E* (Fig. 10). *E* is joined to the pressure equilibrator at tube No. 31, Fig. 1, and otherwise the circuit is the same; channels *A*, Fig. 10, and *B*, Fig. 11, are not used. This procedure is highly desirable for clear observation of blood flow in an organ, for study of rhythmic contraction of smooth muscle and for detecting such abnormal changes as edema, hemorrhage, blood sludging, or clotting. Growth of ovarian follicles with subsequent rupture has been observed under these conditions. However, such a preparation may be expected to show some edema within twenty-four hours. Thomas[4] has expressed the belief that bathing an organ in a continuous flow of freshly aerated blood as well as perfusing it, tends to retard the edema formation. For this reason the apparatus has been constructed in such a way as to permit considerable variation in assembly and use, depending upon the experiment. In its simplest form it has been used to study young surviving isolated rat embryos floating in the organ chamber in blood being circulated through the pump and reservoir only.

Dialyser.—Use of the dialyser is optional, depending upon the nature of the experiment. In accordance with the constituents of the fluid against which the blood is dialysed, certain substances (e.g., urea) may be cleared quite efficiently, and the sugars or other ingredients left within desirable ranges in the blood. By using substances such as dextran, polyvinylpyrrolidone, gelatin, albumin, etc., attempts may be made to remove undesirable accumulations of fluid from the tissues to the blood and thence to the dialysate. Pressures determined by a manometer on each side of the dialysing membrane may be adjusted to permit a differential in one direction or the other. In attempting to eliminate useless or harmful breakdown products, and yet retain desirable levels of nutrients, hormones, antibiotics, vitamins, anticoagulants, etc., the latter may be added by means of the constant feeding device (Fig. 42) or directly and periodically to the reservoir.

Filter.—The importance of having a good filter functioning continuously during a perfusion has been shown by the need to substitute a fresh sheet of cloth after twelve to twenty-four hours of use. A fine cotton broadcloth with 80 by 160 threads to the square inch woven

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Figs. 33-35, Manometer parts Nos. *15* and *16* (Fig. 1). A 2-way stopcock with a metal plate *C* mounted on the top. Plastic tubing *D* containing mercury is held against the plate by spring clamps *E*. The mercury tube goes over the top of the plate at *F* and ends in a length of large plastic tubing sealed at the bottom and used as a trap. Adapter *G* connects the mercury tubing to similar tubing *H* leading to a steel adapter *K* in the head of the stopcock. The stopcock may be turned to tube *A*, Figs. 34, 35, to obtain a reading of the blood pressure in stopcock No. *14* (postfilter) or to tube *B* to read the pressure in the top part of the dialyser (prefilter). Figs. 36 and 37, Flat and cross sectional views of the plastic top or bottom of dialyser. Cellophane membrane *F* is clamped between two such discs as shown in assembly No. 7 (Fig. 1). In the lower disc Tyrode's or other solutions enter the steel tubing at *A* and travels the route indicated by arrows leaving at *B*. In the upper disc blood travels the same route in a countercurrent. Tube *D* connects with mercury manometer No. *9* (Fig. 3) for determining pressure below the cellophane membrane. In the upper disc a similar tube is connected with manometer No. *16*, which may be turned to check the blood pressure in the upper chamber of the dialyser. Through center slot *E* and a hole in the cellophane, a bolt passes. A wing nut on this bolt allows the two plastic discs to be approximated. When they are secured in the brass frames (Figs. 39 and 40) a water-tight seal is achieved. Fig. 38, Plastic top or bottom of filter. A cloth filter is clamped between the two discs using a seal of heavy silicone grease around the periphery of each disc and thus on both sides of the cloth. Blood from the dialyser enters the bottom plastic disc through steel tube *A*, and is forced upward and through the cloth to leave a similar opening diametrically opposite the entrance tube. The baffles, *B*, shown in this disc help to support and tighten the cloth. Fig. 39, Bottom of brass clamp used for dialyser and filter. The plastic discs are compressed between this ring and the upper one (Fig. 40), by means of the swivel bolts with wing nuts. These swing up from the position shown, and engage in the slots in the upper ring. No center bolt is used on the filter. Fig. 40, Flat view of the upper ring of the brass clamp. Fig. 41, Siphon system used to direct dialysing fluid to and from the bottom chamber of the dialyser under a constant head of pressure. These parts are mounted on a wooden frame suspended at the desired height from the wall by block and tackle. Tyrode's or other fluids run from the one-litre bottle *A*, through glass tube *B* into shell vial *C*. The connection is through rubber stoppers. Glass tube *E* carrying plastic tubing *F* into the vial passes through the stopper as shown. There is enough space between the plastic and glass tubing to allow air to enter through *E*. This moves into reservoir *A* in the form of bubbles (*H*) as fluid leaves through *F*. The rate of flow is adjustable by altering the distance between the fluid level *G* in vial *C* and the tip of return tube *K* in vial *L*. A plastic tube adapted at *M* leads to a drain or a bottle for collecting samples for analysis. Fig. 42, A side arm tube for adding White's or other nutrient fluids at a constant rate during the experiment. *A* = 50 ml. tube with side arm capped by rubber stopper *B* pierced by two 17 gauge hypodermic needles. Needle *C* is used to fill or refill *A*. Needle *D* carries plastic capillary tubing *F*, through which the nutrient flows to the main reservoir. The tubing is adapted to a 26 gauge hypodermic needle placed in a drill hole in the side of the reservoir above the blood level (*E*, Fig. 12). Cotton is placed loosely in the two needles as shown (*E*).

in such a way as to minimize the pore size, especially when the cloth is wet, has been satisfactory. Even at the beginning of an experiment, such a filter offers some resistance to the blood flow. This may be checked by turning the main manometer to the proper channels and reading the pre- and postfilter pressures. If the nature of the experiment is such as to cause the filter to clog up slowly, compensations may be made from time to time by raising the prefilter pressure, and finally by substituting a fresh filter.

Interim Modification of the Apparatus.—Since the drawings for this apparatus were made, it has been found that the short lengths of stainless steel tubing placed in the plastic for adapting the plastic tubing may be dispensed with at most points. The plastic tubing is cut to form a taper at one end, and is "threaded" through the plastic channels. This is carried out with the tubing under stretch; and when the tension is released, a snug fluid-tight seal is obtained. Channels are drilled in the plastic in accordance with the caliber of plastic tubing to be used. In order to be able to draw the plastic tubing into the channels in the plastic, smoothly curving channels have been substituted for right-angle bends. This also permits more effective cleansing.

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A RAPID METHOD FOR THE ESTIMATION OF MORPHINE

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INTRODUCTION

A SIMPLE accurate method for the estimation of morphine in body fluids and tissues has not previously been available due to the difficulties involved in the separation and isolation of morphine from the extraneous biologic phase. Many of the older extraction procedures, most of which are modifications of the classical Stas-Otto method,[1] have required the use of time-consuming multiple extractions and solvent evaporations. More recent methods such as adsorption techniques,[2, 3] paper chromatography,[4] and paper ionophoresis[5] still require considerable expenditure of time and/or special laboratory apparatus.

Our study of the partition behavior of morphine has resulted in an improvement in the extraction efficiency of the compound, thus permitting the application of single extraction techniques for the rapid estimation of free and bound morphine. No specialized extraction apparatus is required and a single determination can be carried out in less than ninety minutes.

The procedure as described in this study is based on the photometric estimation of morphine from biologic media as a phenolic compound following its purification and concentration under rigidly defined extraction conditions. Additional directions giving procedures for identifying morphine are also included.

MATERIALS AND METHOD

Reagents.—1. Standard solutions of morphine in concentrations equivalent to 4.0 to 20.0 μg per milliliter of the free base can be made from a stock standard containing 132.9 mg. morphine sulfate·$5H_2O$ made up to 100 ml. with distilled water. 2. Concentrated hydrochloric acid, analytical grade. 3. Potassium hydroxide, approximately 16 N. (Preferably this solution is kept at 50° C. to prevent crystallization.) 4. n-Butanol, analytic grade, saturated with approximately 7 N potassium hydroxide. 5. Sulfuric acid, approximately 0.5 N and 1 N. 6. Phenolphthalein indicator solution. 7. Sodium bicarbonate, analytical grade. 8. Chloroform, analytical grade. 9. Phosphate buffer, pH 5.8 (54 Gm. $NaH_2PO_4 \cdot H_2O$ and 15 Gm. $Na_2HPO_4 \cdot H_2O$ or 25 Gm. $Na_2HPO_4 \cdot 7H_2O$ per liter of solution). 10. Phosphate buffer, pH 6.5 (34 Gm. $NaH_2PO_4 \cdot H_2O$ and 67 Gm. $Na_2HPO_4 \cdot 7H_2O$ per liter of solution). 11. Phosphate buffer, pH 7.6 (7 Gm. $NaH_2PO_4 \cdot H_2O$ and 120 Gm. $Na_2HPO_4 \cdot 7H_2O$ per liter of solution). 12. Silicomolybdic acid reagent made according to Snell and Snell.[6] 13. Ammonium hydroxide concentrated, analytic grade.

a. *Extraction procedure for urine: Total morphine,* (bound plus free). Acidify 15 ml. of urine with 1.5 ml. concentrated hydrochloric acid in a 50 ml. glass-stoppered centrifuge tube. Stopper and autoclave for 30 minutes at 15 to 20 pounds pressure.

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Cool the hydrolysate, add 10 ml. 16 N potassium hydroxide and 20 ml.* of n-butanol. Stopper and shake the mixture for 5 minutes, preferably in a mechanical shaker. Centrifuge for one minute at 2,000 r.p.m. and transfer a 15 ml. aliquot of the butanol layer to a centrifuge tube containing 22 ml.* of 1 N sulfuric acid. Shake for 3 minutes and centrifuge. Remove the butanol layer by aspiration and transfer 20 ml. of the acid layer to another extraction tube containing a drop of phenolphthalein indicator. While agitating the tube, add concentrated potassium hydroxide until a definite pink color is obtained (approximately 1.2 ml. of alkali). Add approximately 1 Gm. of sodium bicarbonate and shake until dissolved. Add 21 ml.* chloroform and shake for five minutes. After centrifugation, aspirate the upper aqueous layer. Shake a 20 ml. aliquot of the chloroform layer in a centrifuge tube with 7 ml. of phosphate buffer, pH 5.8. After centrifuging, transfer 5 ml. of the final buffer solution of morphine to a 10 ml. volumetric flask; to this aliquot add 2 ml. of silicomolybdic acid reagent and 2 ml. concentrated ammonium hydroxide. Immediately stopper and shake; bring up to volume with distilled water. Immediate mixing is necessary to prevent formation of small amounts of precipitate. Allow 15 minutes for full color development and determine the optical density at 675 mμ on a spectrophotometer using distilled water for zero setting. After correcting for reagent blank (0.018 O.D. unit) the amount of morphine is obtained from a standard curve for morphine.

Free morphine: Omit the hydrolysis step and continue with the alkalinization and butanol extraction. The total minus the free morphine gives the bound morphine.

b. *Extraction procedure for liver:* With some slight modifications, the extraction procedure used for urine has been incorporated into the procedure for morphine in the liver. For total morphine, mince 30 grams or more of liver in a Waring Blendor with an equal weight of water; tare four 50 ml. centrifuge tubes and weigh 10 grams of this homogenate into each tube, thus making a 20-gram aliquot of the original liver sample. Add 2 ml. of 40 per cent trichloroacetic acid and mix thoroughly. Add 1.5 ml. of concentrated hydrochloric acid to each tube, stopper, and autoclave as for urine. Extract the hydrolysate with 20 ml. of butanol after adding 10 ml. of 16 N potassium hydroxide. Centrifuge and take a 15 ml. aliquot of the butanol from each tube and combine these aliquots in a larger centrifuge tube containing 22 ml. of 1 N sulfuric acid. Shake, then centrifuge. Transfer 15 ml. of the sulfuric acid layer to another centrifuge tube, neutralize to phenolphthalein with 16 N potassium hydroxide, add bicarbonate, and extract with chloroform following the corresponding steps as for the urine from here on.

Application of the Specificity Tests.—Although the specific extraction steps for morphine eliminate virtually all of the naturally occurring phenolic substances which may react with the silicomolybdic acid reagent, therapeutic agents possessing structural features common to morphine may interfere with its determination. To appraise the specificity of the method for morphine the partition behavior and ultraviolet absorption properties of the compound were studied.

A. *Partition behavior:* The probability of two different organic bases having identical partition ratios between two immiscible solvents under different pH conditions is extremely remote. Specificity of method, therefore, can be appraised by comparing the distribution characteristics of recovered and added morphine between the chloroform and phosphate buffers of varying pH. The procedure described for morphine can easily be adapted for such purposes in the following manner:

The extraction procedure is carried out in quadruplicate. In the last extraction step of the procedure described previously, where 20 ml. of the chloroform layer is shaken with 7 ml. of 0.5 M phosphate buffer, pH 5.8, equilibrate each of the three additional chloroform extracts in a similar manner with pH 6.5 and 7.6 phosphate buffers and with 0.5 N sulfuric acid. The morphine in the aqueous layers is then determined in the usual manner. Assuming complete extraction with the sulfuric acid, the fraction of the silicomolybdic acid reactants extracted by each of the buffers as compared with sulfuric

*Addition of solvents can be greatly facilitated by using stop syringe pipettes, available from Alfred Bicknell Co., Cambridge, Mass.

acid is expressed as a ratio, $\frac{\text{O.D. reading of buffer}}{\text{O.D. reading of acid}}$. The ratios thus obtained serve to identity the product as morphine.

B. *Ultraviolet absorption characteristics:* The final buffer extract of morphine obtained from the extraction procedure can also be used in ultraviolet absorption studies to characterize morphine, since the compound yields characteristic ultraviolet absorption curves under acidic and alkaline conditions. Pipette 3 ml. of the pH 5.8 buffer extract into the quartz cell of a Beckman DU spectrophotometer and take the optical density readings at 5 mμ intervals from 310 to 230 mμ wave length, using pH 5.8 buffer for zero settings. Repeat the procedure after adding 0.5 ml. of 50 per cent potassium hydroxide to each cell and mixing thoroughly. Subtract the acid (buffer) optical density readings from 1.17 times the basic O.D. readings (corrected for dilution) to obtain a difference curve.

RESULTS

Table I summarizes the data on the recovery of morphine added to twenty different urine samples. Determinations were made in duplicate and found to agree within ± 0.01 optical density. Blank values for free and total morphine are also listed. Free phenolic blanks averaged .006 ± 0.002 O.D. unit; total phenols 0.025 ± 0.012. The maximum error at concentrations greater than 6 μg per milliliter was found to be less than ± 10 per cent. This degree of sensitivity and precision is usually adequate for clinical and forensic application.

TABLE I. BLANKS AND STANDARD RECOVERIES FOR MORPHINE ADDED TO URINE

URINE SPECIMEN	BLANK OPTICAL DENSITY		MORPHINE ADDED (MICROGRAMS/ML.)	RECOVERY OF MORPHINE (%)	
	FREE	TOTAL		FREE	TOTAL
E. W. ♂	.005	.013	3	94.2	94.4
H. T. ♂	.006	.018	3	97.6	111.4
D. F. ♀	.003	.023	3	104.0	104.7
J. H. ♂	.004	.018	3	109.0	112.0
J. C. ♀	.003	.005	6	108.2	106.2
M. H. ♂	.005	.033	6	109.0	108.5
A. T. ♂	.005	.020	6	103.4	107.0
T. A. ♀	.006	.033	6	102.0	104.7
D. S. ♀	.007	.035	6	105.5	100.4
N. F. ♀	.006	.033	9	98.4	95.9
C. C. ♀	.015	.045	9	101.2	100.4
J. M. ♂	.006	.035	9	95.6	99.6
G. K. ♂	.005	.026	9	101.2	101.7
C. S. ♂	.003	.015	9	102.0	101.7
G. M. ♂	.010	.045	9	104.0	98.8
V. S. ♀	.005	.018	15	97.0	103.2
E. R. ♀	.005	.015	15	97.0	97.3
M. N. ♂	.003	.020	15	95.0	99.6
J. F. ♂	.005	.023	15	101.2	104.7
J. K. ♂	.005	.025	15	107.0	99.6
Mean =	.0056	.0245			
S.D. ±	.002	± .012			

Recovery of morphine added to liver (5 to 15 μg of drug per gram wet tissue) was approximately ninety per cent. While the previously mentioned order of sensitivity may be sufficient in some instances to establish morphine overdosage, the usefulness of the procedure would be enhanced greatly if

smaller quantities of morphine could be detected. Only limited studies have been carried out to date with respect to improving sensitivity; however, the problem is under consideration.

Both the partition behavior and ultraviolet absorption experiments gave evidence that the specific conditions of extraction convey a high degree of specificity for morphine. With respect to the partition studies, the results in Table II indicate that the partition ratios of added and recovered morphine agree within limits of experimental error. Since it was not possible to actually measure the blank contribution in the recovery samples, a mean blank correction (0.05 O.D. unit for 0.5N H_2SO_4; 0.025 O.D. unit for each buffer) was applied in calculating the ratios for total morphine.

TABLE II. EFFECT OF PH UPON THE PARTITION OF VARIOUS COMPOUNDS FROM CHLOROFORM, USING 0.5 N H_2SO_4 AS INDEX (1.00)

COMPOUND IN URINE	FRACTION EXTRACTED FROM $CHCl_3$ AT PH: 7.6	6.5	5.8
Morphine (added)	.29	.77	.94
Free Morphine	.34	.74	.91
Total Morphine (recovered from urine No. 2387)	.35	.77	.94
Total Morphine (recovered from urine No. 212076)	.31	.82	.95
Nalorphine (added)	.01	.10	.29
Dihydromorphinone (added)	.05	.38	.68
Metopon (added)	0	.12	.21
m-Aminophenol from PAS (added)	.54	.56	.80

The partition ratios obtained with other compounds closely related to morphine or with similar functional groups which may interfere with the method are also included in the table for comparison. It is evident that none of the ratios of these compounds resembles those obtained with morphine.

The extraction procedure also reduces blank contribution sufficiently to enable the application of ultraviolet absorption techniques for identifying morphine. In accordance with Goldbaum and Kazyak[5] a difference curve for morphine in the ultraviolet, obtained by subtracting optical density readings of morphine in acid solution (pH 5.8) from those in alkali, was found to have maxima at 255 and 300 mμ and a minimum at 280 mμ for both morphine in pure solution and recovered from the urine of man receiving the drug. Naturally occurring substances in urine do not seriously interfere with the identification of morphine but further studies are necessary before the ultraviolet procedure can be used for quantitation of morphine. Fig. 1 gives the difference curves obtained with a sample of blank urine and with the same urine after addition of morphine in final concentration of 9 μg per milliliter. Fig. 2 shows the difference curves for hydrolyzed urine of two recovery samples which have maxima and minima corresponding to those of morphine added to urine.

The two recovery samples used in the afore-mentioned studies to illustrate the specificity of the morphine method were urine specimens obtained from two cases of morphine poisoning referred to us for analysis. Case No. 2387

was an attempted suicide and, according to the attending physician, a morphine addict. Analysis of the urine for morphine indicated the presence of 14 mg. of free morphine and 303 mg. in the bound form. Case No. 212076 was found to have 2.3 mg. of free morphine and 13.8 mg. of bound morphine in the urine. In both instances morphine was easily identified and quantitated.

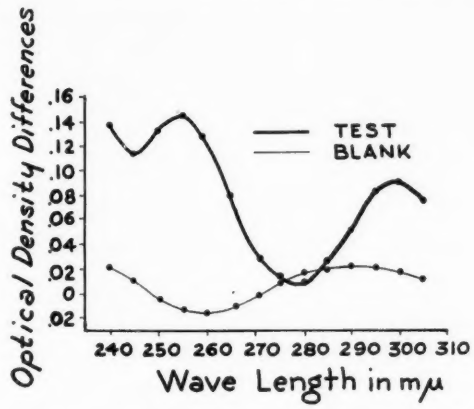

Fig. 1.—Ultraviolet difference curves for extracts from blank urine and the same urine with 9 μg of morphine added per milliliter.

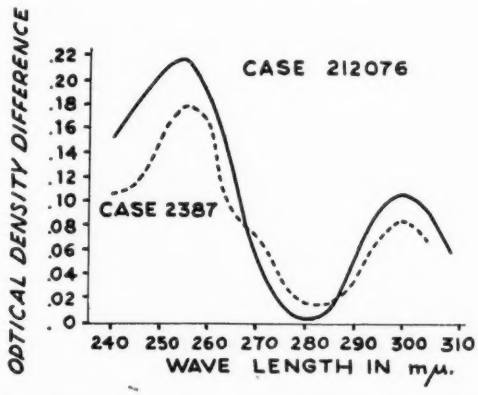

Fig. 2.—Ultraviolet difference curves for unknown urines analyzed for presumptive morphine poisoning.

DISCUSSION

A general consideration of the factors which finally led to the extraction procedure is essential for a full comprehension of the method.

Numerous reagents are available which give color reactions with morphine. The Snell silicomolybdic acid phenolic reagent in accord with Shideman and Kelley[7] was found to yield a reliable color reaction for the quantitative estimation of morphine. The feasibility of using another phenolic reagent, such as the Folin-Ciocalteau reagent, for increasing the sensitivity of the method for morphine was tested and subsequently rejected because of high urine blanks. Even with the silicomolybdic agent, direct color development with a 15 ml. urine sample as used in the procedure yielded reactants

equivalent to about 30 mg. of morphine. The main problem, therefore, was to establish conditions which would eliminate these interfering substances and yet facilitate the efficient extraction of morphine from biologic media. The initial extraction with butanol from strongly alkaline solution largely accomplishes both factors in a single step. Analysis of the sulfuric acid extract of the butanol layer indicated the presence of 85 per cent of the morphine and only 0.7 per cent of the original blank values. The partition ratios for all the subsequent extraction steps are at least as favorable. The over-all recovery was calculated to be 71 per cent for free morphine and 68 per cent when determined as total morphine.

TABLE III. EFFECT OF CONCENTRATION OF ALKALI ON MORPHINE EXTRACTION BY BUTANOL

ADJUSTED URINE NORMALITY	BASE USED	PER CENT EXTRACTED
0.4	NaOH	45
2.0	KOH	50
2.5	NaOH	42
4.0	NaOH	50
5.0	NaOH	80
7.2	KOH	86
8.0	KOH	88

The extraction of morphine by the butanol is dependent in part on the concentration of alkali added to the urine. Table III shows that as the concentration of alkali is increased the extraction of the morphine is increased.

The increased extractability using high alkali concentrations is probably a salt effect of inorganic ions, since the addition of sodium chloride to the urine increased morphine extraction for a given alkali concentration.

Numerous attempts to further reduce the blank interference of the butanol extract of urine in a single step without a concomitant loss in morphine were not successful. These included preliminary extraction of the acidified urine with organic solvents, washing the alkaline morphine-containing butanol layer with various buffers, and use of various adsorbing agents. Consequently, the sulfuric acid was neutralized and the additional extraction step with the chloroform was instituted.

In the neutralization step of the sulfuric acid, concentrated potassium hydroxide is added until alkalinity to phenolphthalein is achieved. Addition of sodium bicarbonate then effects a suitable pH adjustment as indicated by the disappearance of the pink color. This procedure brings the pH of the solution into the region of the isoelectric point[8] for morphine (pH 8.4 to 9.4) which facilitates its extraction by chloroform. In this step about 85 per cent of the morphine is partitioned into the chloroform. The presence of small amounts of butanol carried through the procedure apparently enhances the extraction of morphine by chloroform since extraction with chloroform of morphine added to water or urine under otherwise identical conditions removes only about 50 per cent of the compound. Thus, the butanol and chloroform extraction stages account for almost the entire recovery loss in the procedure.

In the final extraction of the chloroform aliquot, the pH of this buffer is important, as shown in Fig. 3. The blank values of the hydrolyzed urine are constant and minimal at pH 5.75 to 9.5, but rise at pH 0.7 (sulfuric acid) and 12.5 (sodium hydroxide). The partition of morphine into the aqueous phase as shown by the solid bars increases with decreasing pH but is practically complete between pH 5.75 and 5.9 where the blank is low (free phenol, 0.5 μg; total phenol, 2 μg). These findings provide the rationale for using the pH 5.8 buffer for the quantitation of the morphine and the basis for subsequent appraisal of specificity of the method.

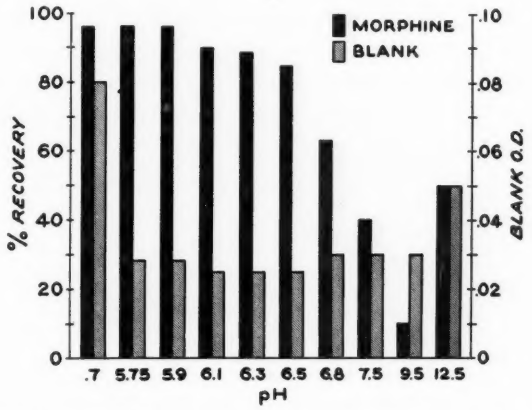

Fig. 3.—Relation of pH to blank interference and morphine recovery.

Certain medicinal agents with structural features common to morphine may yield false positive or high values for morphine, particularly if the final extraction step of the method is performed only with buffer pH 5.8. Compounds which may give rise to erroneous results to a varying degree include: N-allyl normorphine (nalorphine or Nalline); the closely related analgetics, such as Dromoran (3-hydroxy-N-methyl morphinan), dihydromorphinone (Dilaudid), and Metopon; normorphine, a possible metabolite of morphine; and the tuberculostatic agent, p-aminosalicylic acid (PAS). Although PAS itself is not carried through the procedure, if present, it is decarboxylated upon heating (the hydrolysis step) to m-amino phenol which is then extracted in part and reacts with the silicomolybdic acid reagent. Differentiation of these substances from morphine by either the partition behavior or ultraviolet absorption characteristics is not too difficult, but precise estimation of morphine in their presence is more complicated.

Nalorphine can easily be mistaken for morphine. Its increasing use as an antidote for ovedosage by morphine-type compounds will complicate the identification and quantitation of morphine. Ultraviolet absorption studies have limited application since morphine and nalorphine have as a common ultraviolet chromophore, the hexahydrophenanthrene ring, and as a result exhibit almost identical absorption curves.[12] The difference in partition behavior as brought out in the extraction procedure serves to distinguish mor-

phine from nalorphine but in a mixture of the two substances to obtain valid data for morphine a correction must be applied to the morphine values taking into account the nalorphine (29 per cent) carried through the procedure. It is necessary, therefore, in carrying out morphine determination to have the information whether nalorphine had been administered for antidotal purposes.

Normorphine also has the same type of ultraviolet absorption spectra as morphine and gives silicomolybdic acid color intensities comparable to morphine when reacted directly with the reagent, but only approximately 10 per cent is carried through the extraction method. Normorphine has not been conclusively established as a morphine metabolite, but the possibility exists since normeperidine[9] and norcodeine[10] have been characterized as metabolites of meperidine and codeine, respectively. However, since Elliott and associates,[11] report that less than 6 per cent of $N\text{–}C^{14}H_3$ labeled morphine is excreted as $C^{14}O_2$, it would appear that only minor amounts of normorphine are formed and the compound should not interfere seriously in the morphine determinations.

Dromoran (3-hydroxy-N-methyl morphinan) is carried to the final buffer layer to a considerable extent, but its color index with the silicomolybdic is too low to be of consequence. Moreover, its ultraviolet characteristics are considerably different from those of morphine.

It is not possible to quantitate morphine accurately in the presence of Dilaudid and Metopon by the present method. Both compounds are carried through the extraction procedure in appreciable quantities and give a blue color with silicomolybdic acid, although the color is only about one-half the intensity obtained with morphine. However, the necessary differentiation from morphine can be established easily since their partition ratios (Table II) and ultraviolet curves differ sufficiently from those of morphine. The ultraviolet difference curves for both Dilaudid and Metopon peak at 240 mμ instead of 255 mμ as for morphine, and similar though smaller differences can be noted at other points.

The presence of m-aminophenol in the urine of tubercular patients on a regimen of PAS must always be considered. It can easily be distinguished from morphine by applying the comparative ratios and ultraviolet difference curve procedure, but means must be developed to eliminate its interference with morphine determinations.

SUMMARY

A rapid extraction method for the photometric estimation of morphine in biologic media is described. The method utilizes single extraction techniques and has a high degree of specificity for morphine. Considerable time saving is accomplished by extracting morphine from a highly alkalinized solution with n-butanol to give the major separation of the morphine from the extraneous interfering material. Subsequent extraction steps remove further interfering substances without undue loss in time or yield of morphine. A colorimetric phenol reagent is used for the final estimation of the morphine in the

buffer extract. A highly specific test for morphine is also obtained by simply incorporating the ultraviolet absorption and partition ratio determinations into the general procedure. Two actual cases of morphine poisoning were established by the use of the developed method.

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PAPER ELECTROPHORESIS WITH AUTOMATIC SCANNING AND RECORDING

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PAPER electrophoresis permits simultaneous analyses of the protein composition of several blood sera and other body fluids by means of a relatively simple and inexpensive apparatus. Gross deviations from the usual proportions among serum protein fractions, as well as the presence of abnormal proteins, can be detected by direct visual inspection of the stained paper pattern.[1-4] More precise quantitative analysis is possible by one of several methods, e.g., nitrogen determination in sections of the stained paper strip,[5] photometric measurement of the stain eluted from such sections,[2, 3, 6-12] or direct scanning of the whole strip in a photometer (densitometer).[13-19] In either of the two latter instances, the galvanometer readings obtained are usually recorded and plotted against corresponding distances on the paper strip for the purpose of area measurements. Obviously, the scanning procedure is superior in practicality and economy of time. These qualities would be further enhanced by the automatic graphic recording of the galvanometer deflections.[13, 14, 18, 19, 20] In the present paper, an automatic scanning and recording apparatus will be described that furnished electrophoretic patterns closely comparable to those obtained by means of the standard Tiselius method of moving-boundary electrophoresis.

EQUIPMENT AND METHODS

A bridge-type paper electrophoresis apparatus was used which conformed in principle to Kunkel and Tiselius' description[7] (Fig. 1). Simultaneous runs on six serum samples, each 0.005 ml. in volume, were usually performed, using two three-inch-wide strips of Whatman No. 3 or 3M filter paper. After application of a constant direct current (approximately 75 v. and 5.5 ma.) for sixteen hours (overnight)—resulting in migration of the albumin component over a distance of about 10 cm. from the origin—the paper strips were, without drying, fixed and stained for five minutes in an alcoholic saturated mercuric bichloride solution containing 1 per cent bromphenol blue, and then washed in 0.5 per cent acetic acid eight times for five minutes each. The air-dried stained strips (paper electrochromatograms) were exposed to ammonia for intensification of the stain immediately before being subjected to either elution or scanning analysis.

For the purpose of this study, elution was applied after the stained paper strip had been divided into two segments only, one containing the albumin and the other exhibiting all the globulins. Following exposure of each segment to 0.01 N NaOH[7] for one hour, optical density readings of the respective eluates were obtained in a Coleman Model 6A

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spectrophotometer;* from their ratio the albumin values in per cent of total protein were calculated.

For direct scanning of the paper electrochromatograms, the Ansco Color Densitometer Model 12 was selected (Fig. 2),† chiefly because of the logarithmic electrical response which it furnishes and the consequent remarkable linearity that was found to obtain between optical density readings and protein concentrations on the paper strip. The electron multiplier phototube of the apparatus is combined with a dynode voltage feedback arrangement, permitting a practically instantaneous electrical response that shows no fatigue and is therefore free from oscillation. The light intensity could be so increased as to permit, with use of the green filter (maximum at 546 mμ), a zero optical density scale reading on the filter paper, pretreated with mineral oil, when the round light beam measured 2 mm. in diameter on the paper—conditions which were found optimal for routine scanning.

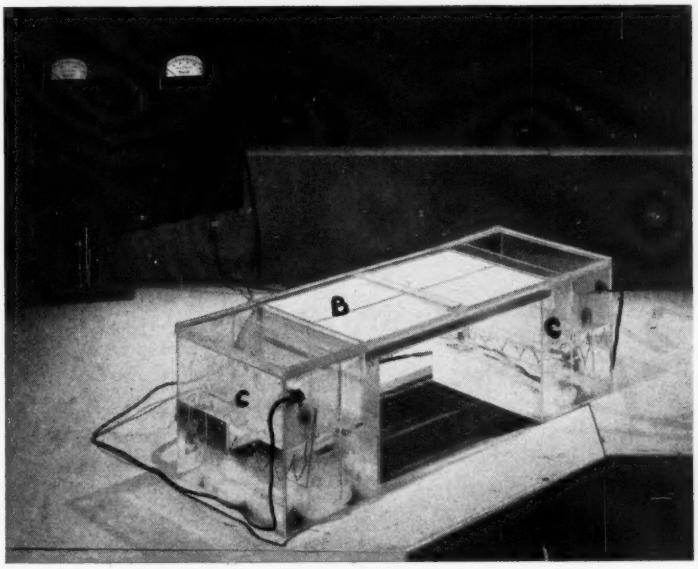

Fig. 1.—Paper electrophoresis apparatus. Filter paper strips placed on glass plate of bridge (*B*) dip into barbital buffer of pH 8.6 and 0.1 ionic strength, contained in plastic electrode compartments (*C*). Central transverse slot in elevated plastic bridge top (provided with lid) serves for application of protein specimens after fluid equilibrium is attained in paper. Evaporation is controlled by sealing with cellophane tape.

Automatic recording (Fig. 2) was accomplished by means of a modified Sanborn electrocardiograph (Model 127 industrial recorder).‡ The necessary intensification of the current from the densitometer was secured through a Sanborn D.C. amplifier (Model 126) and a D.C. preamplifier (Model 60-400). Three voltage transformers (Model 30806, capacity = 1.04 amperes) were required for stabilization of current and avoidance of undue fluctuations in the recorded patterns. A standard Aminco-Stern apparatus was used for boundary-type electrophoresis, in accordance with techniques previously described.[22]

CONTINUOUS SCANNING AND AUTOMATIC RECORDING PROCEDURE

A stained paper strip is exposed to ammonia, dipped in mineral oil, carefully blotted to remove excess oil,§ and then inserted into an aluminum frame. The latter fits into a run-

*Trade names are used throughout as a means of identifying the products under discussion and do not represent endorsement by the Public Health Service.

†This instrument had originally been designed for use in motion picture work.[21]

‡An ordinary electrocardiograph can be used, supplemented with a direct current preamplifier, or else other types of automatic recorders.[21]

§Vacuum treatment of the oiled strip, recommended by Grassmann and Hannig,[14] with a view of eliminating optically active air bubbles, was not adopted by the authors because of satisfactory reproducibility obtained without this complicating step.

way, handmade of cardboard, placed on top of the densitometer and beneath the photocell, so that the center of the runway's width coincides with the light beam. Under the arrangement depicted in Fig. 2, the right side of the sliding frame is hooked to the graph paper coming from the recorder, while weights are applied to the frame's left end by a string carried over a pulley. The constant pulling force thus exerted simultaneously upon both the frame containing the paper electrochromatogram and the graph paper causes the frame to slide (from right to left) at exactly the same rate at which the graph paper emerges from the recorder when the recorder's motor is on. The weights must, of course, be so selected as to ensure constant tautness of the graph paper without accelerating the original recorder speed. Before a recording is made, both apparati must be turned on for at least fifteen minutes. The densitometer is standardized by obtaining a zero scale reading from portions of the chromatogram that show no protein staining. Planimetry of areas under different peaks of the recorded pattern (paper electropherogram) furnishes values for corresponding protein fractions in per cent of total protein.*

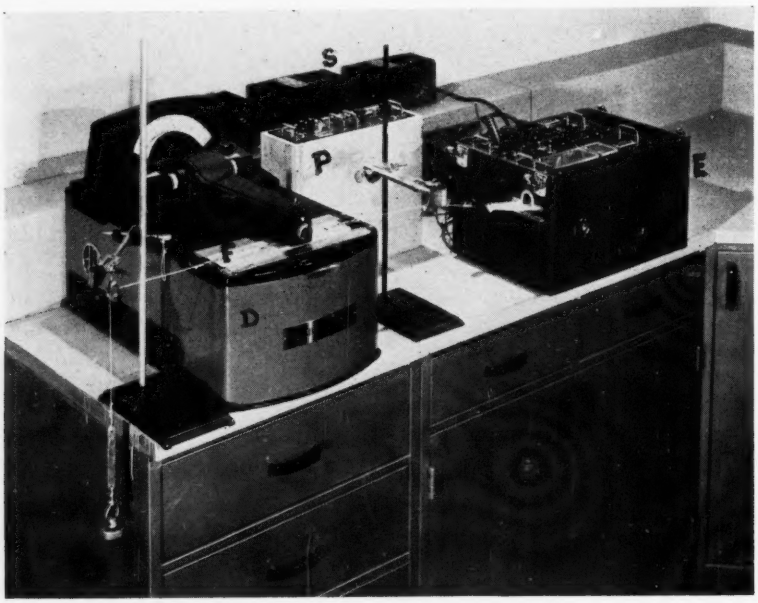

Fig. 2.—Automatic scanning and recording apparatus, including densitometer (*D*), electrocardiograph with D.C. amplifier (*E*), preamplifier (*P*), voltage stabilizers (*S*), sliding frame for stained paper strip (*F*) with string, pulleys, and weight. (See text.)

RESULTS

A typical paper electropherogram obtained by means of the scanning and recording equipment is seen in Fig. 3, along with the corresponding stained paper strip and an electrophoretic pattern secured by the classical boundary-type method. Calculated values for protein fractions derived by the two methods from sera of twelve healthy subjects (Table I) showed differences which were not inconsiderable. With respect to the albumin component, which may be most suitable as an index of comparison, the boundary method yielded higher values in the majority of instances. The mean paper albumin value for the group was 54.0 per cent of total protein (T. P.) with the scanning and recording procedure and 57.0 per cent T.P. with use of the elution technique,

*Weighing of the cutout areas or counting of the minute squares of the graph paper included in each area may be substituted for instrumental planimetry.[16]

as against the mean boundary albumin value of 57.8 per cent T.P. Among the globulins, it was the gamma fraction which deviated most in the (scanning) paper electropherograms from the corresponding boundary value by being, on the average, 5.2 per cent T.P. higher, chiefly at the expense of the albumin and beta fractions. The mean difference (m) between boundary and paper values for albumin was 3.4 per cent T.P. when the latter were secured with the scanning-recording device, and 0.9 per cent T.P. when the paper values were obtained by elution; the standard deviations (s) of these respective mean differences were practically identical, i.e., 3.27 and 3.25 per cent T.P.[23] Hence, standard boundary albumin values (A^S) might be predicted from corresponding paper values (A^P) by the formula $A^S = A^P + m \pm t\ s$,* if statistical validity can be attached to the small number of samples considered.

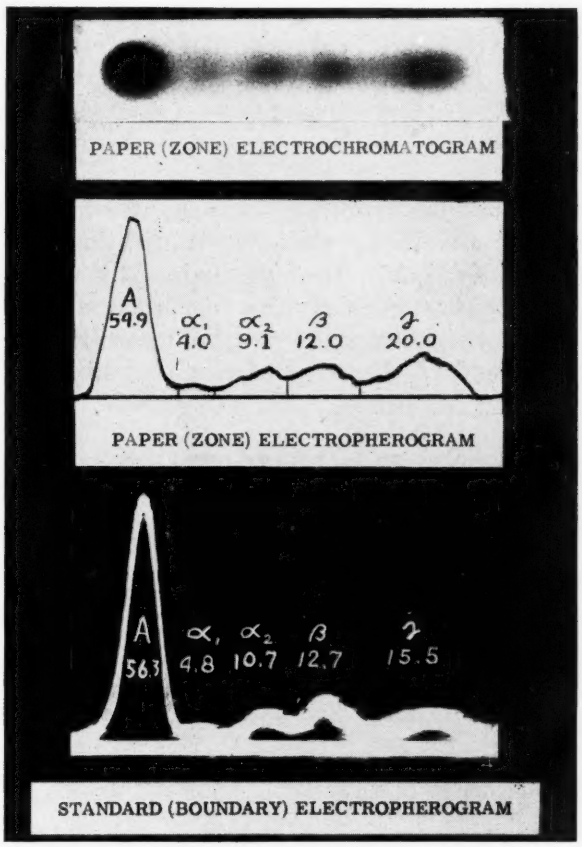

Fig. 3.—Comparison of paper and boundary electropherograms from normal human serum.

The fact that the differences between results of paper (scanning) and of boundary electrophoresis were statistically significant, though small numerically, was emphasized by an analysis of variance of albumin values (Table II, B), which included consideration of the experimental error inherent in each

* If t = 1 (0.67 probability level), the value for A^S may be expected to lie between A^P + 0.1 and A^P + 6.64 per cent T.P. when A^P is derived by means of scanning, or between A^P − 2.35 and A^P + 4.15 when A^P is determined by elution.

TABLE I. COMPARISON OF RESULTS OF PROTEIN FRACTIONS AS OBTAINED BY BOUNDARY AND PAPER ELECTROPHORESIS UPON TWELVE NORMAL SERA

	PER CENT TOTAL PROTEINS										
	BOUNDARY ELECTROPHORESIS					PAPER ELECTROPHORESIS					
						SCANNING-RECORDING					ELUTION
SERUM NO.	ALBUMIN	GLOBULINS α_1	GLOBULINS α_2	GLOBULINS β	GLOBULINS γ	ALBUMIN	GLOBULINS α_1	GLOBULINS α_2	GLOBULINS β	GLOBULINS γ	ALBUMIN
1	53.9*	4.2	9.0	13.2	19.7	51.9	3.6	9.1	10.9	24.5	52.4
2	54.8*	5.8	11.6	15.6	12.2	53.4	4.4	12.3	12.7	17.2	59.6
3	55.9	4.2	10.4	14.3	15.2	48.3	3.1	13.2	14.2	21.2	52.4
4	56.2*	4.1	7.9	15.5	16.3	53.2	4.1	7.8	13.1	21.8	58.3
5	56.3*	4.8	10.7	12.7	15.5	54.9	4.0	9.1	12.0	20.0	52.6
6	56.8*	4.5	10.4	14.9	13.4	55.0	3.6	10.9	11.4	19.1	58.7
7	58.0*	5.6	10.7	15.5	10.2	59.8	4.2	8.9	13.5	13.6	59.7
8	58.5	4.8	8.3	14.3	14.1	52.2	4.2	11.0	12.3	20.3	54.1
9	59.3	4.6	9.1	13.0	14.0	54.2	3.7	11.3	11.7	19.1	59.5
10	59.4	6.1	9.5	13.5	11.5	51.4	4.4	11.5	14.2	18.5	57.9
11	61.3	4.5	9.3	12.8	12.1	56.6	3.7	10.9	12.6	16.2	56.7
12	62.9*	4.9	9.3	13.3	11.6	57.6	4.3	8.0	13.5	16.6	61.6

Results of boundary electrophoresis were calculated as the means of ascending and descending patterns. All protein values listed are means of replicate (duplicate and triplicate) measurements, except for the boundary values corresponding to *, which represent single runs. (Individual replicate values were used for computation of means and standard deviations quoted in text.)

method. This error (among replicates) was greater in the former method (s = 1.8 for scanning and 1.5 for elution), than in the boundary procedure (0.6 per cent T.P.) (Table II, A). The magnitude of the variation encountered with paper electrophoresis was chiefly due to the preparation of the electrochromatogram. Among eight paper electropherograms secured from a single chromatogram, the standard deviation of albumin values was only 0.6 per cent T.P.

TABLE II. VARIATION OF ALBUMIN VALUES OBTAINED FROM SERA OF TWELVE NORMAL SUBJECTS BY BOUNDARY AND PAPER ELECTROPHORESIS

A. DERIVATION OF STANDARD DEVIATION

	BOUNDARY ELECTROPHORESIS				PAPER ELECTROPHORESIS							
					SCANNING-RECORDING				ELUTION			
Source of variation	S. S.	D. F.	s^2	s	S. S.	D. F.	s^2	s	S. S.	D. F.	s^2	s
Subjects	98.11	11	8.91	2.99	212.70	11	19.34	4.40	242.54	11	22.05	4.70
Serum replicates	1.88	6	0.31	0.56	46.71	14	3.34	1.83	25.52	11	2.32	1.52

B. ANALYSIS OF VARIANCE FOR BOUNDARY AND PAPER METHODS

	BOUNDARY VS. SCANNING-RECORDING				BOUNDARY VS. ELUTION			
Source of variation	S. S.	D. F.	M. S.	F-RATIO	S. S.	D. F.	M. S.	F-RATIO
Methods	145.11	1	145.11	33.67*	11.72	1	11.72	3.56^{NS}
Subjects	225.68	11	20.52	4.76*	276.03	11	25.09	7.63*
Error†	133.71	31	4.31	--	92.01	28	3.29	--

S.S. – Sums of squares of deviation from the mean.
D.F. – Degrees of freedom.
s^2 – Estimates of variance.
s – Standard deviation among replicates (in per cent of total protein).
M.S. – Mean squares.
* – Statistically significant at the 0.001 probability level.
NS – Statistically not significant.
† – Total variation less variation attributable to differences between methods and subjects. (Subject – method interaction was not computed because of inequality of subclass numbers.)

DISCUSSION

The ranges and means of the various protein fractions observed in our series of twelve normal sera by means of the standard boundary electrophoresis (Table I) conform to corresponding values reported by others.[8, 11, 14, 22, 24, 25] While the values derived from paper electrochromatograms by the scanning-recording procedure differed somewhat from corresponding results furnished by the previously mentioned method, especially by showing lower albumin and higher gamma globulin fractions, they agree closely with boundary results contained in at least *one* report upon a larger series of normal sera.[24] Furthermore, they were well reproducible. The analytic error encountered (s = 1.8 per cent T.P.) is small indeed when considered in relation to the minute volume of serum (0.005 ml.) being analyzed. Of the same order of magnitude were the errors observed by Plückthun and Götting[8] with the elution procedure and by Grassmann and Hannig[14] and Sommerfelt[15] who used the direct scanning principle, viz., 1.2, 1.2, and 1.6 per cent T.P., respectively. From the statistical data of Grassmann and Hannig,[14] as well as from ours, it is evident that more than one-half of the error must be attributed to the processes involved in producing the electrochromatogram; the combined photometric-planimetric measurement accounted for an error of only 0.4 per cent T.P. in Grassmann and Hannig's and of 0.6 per cent T.P. in the present work.

The elution results for albumin observed in the present study did not differ significantly from albumin values furnished by boundary electrophoresis (Table II, B). This finding is in accord with similar comparisons by other workers,[2, 7-12] although their elution results, when based on bromphenol blue as a stain,[2, 7, 9, 10, 12] required the use of correction factors because of apparent disparities in affinity between dye and different protein fractions. Our experiences with the elution technique, which involved many more serum samples than are being considered here and which will be reported separately, indicate a far more erratic behavior than is evident here and tend to confirm the vagaries observed by others.[26, 27] The amount of time spent in sectioning, eluting, and photometric reading would seem to deter the use of this method in routine work, unless sectioning into the main protein fractions only is adopted.[5, 6, 8, 9, 11]

When using the multiple-sectioning method,[2, 3, 4, 7, 10, 12] only four separate electrochromatograms could be carried through the photometric and plotting steps by one of the authors in an eight-hour day. Manual scanning by means of the densitometer permitted the drawing of electrophoretic curve patterns of four chromatograms in about one hour. With use of the electrocardiograph attached to the densitometer as described, the electropherograms of fifteen to twenty different chromatograms could be conveniently recorded in one hour

An automatic scanning and recording device applied to paper electrophoresis has been briefly reported by Crook and co-workers.[18] These authors used an apparatus which included a synchronous electric motor that moved the paper strip (dyed with azocarmine B) between an illuminated slit and a photocell. The current from the photocell was amplified and recorded with a pen-recorder. Absence of linearity between optical density and protein concentration on the paper strip required special calibration of the instrument

with serial protein dilution. A semiautomatic recording densitometer has been devised by Latner and associates[19] which utilizes the principle of reflection scanning. Movement of the (naphthalene black) stained paper strip across the light beam, regulated through the medium of a hand-operated screw, was stopped at brief intervals in order to allow a spring-loaded pen to be pressed against graph paper. Connection of all the ink dots thus produced resulted in a typical electrophoresis curve. The accuracy of results was checked by means of prepared mixtures of nearly pure serum protein fractions. As many as ten paper strips could be scanned in an hour. Other literature references to automatic recording of paper electrochromatograms do not give any details.[13, 14]

The discrepancy encountered by us between results of paper electrophoresis and those of the classical boundary technique is by no means unexpected. In the latter method, substances other than proteins, especially lipids, contribute to the formation of boundaries between protein fractions, while in filter-paper electrophoresis only the proteins combine with the stain and are thus measured[3, 7, 8, 11, 14, 19]; hence, the beta globulin is usually lower (Table I). Furthermore, absolute separation of components takes place on the filter paper, in contrast to separation into concentration gradients by free (boundary) electrophoresis.[7] The disparity observed in sera from normal subjects may conceivably be augmented in pathologic sera.[2, 3, 7] It would seem that paper electrophoresis should be regarded as a specific technique all its own[28] which furnishes normal serum protein values within a certain range and can thus be utilized for recognition of abnormalities in the protein pattern due to disease.

The advantages of the automatic scanning-recording equipment described previously in this article include rapidity of operation, an acceptable normal range, good reproducibility and accuracy, commercial availability, and prompt utilization without special calibration. In addition, the method was found to reflect reliably deviations of the serum protein fractions from the normal range in a variety of diseases in over 200 hospital patients, experiences which will be the subject of a subsequent report.

SUMMARY

Combining a densitometer with an electrocardiograph was found to lend itself to the automatic scanning of filter-paper strips containing electrophoretically separated and stained serum proteins and to the simultaneous recording of typical electrophoresis curves. Differences in numerical values obtained in twelve normal sera by paper and boundary electrophoresis were small but statistically significant; with the paper method the albumin was, on the average, lower by 3.8 per cent of total protein and the gamma fraction higher by 5.2 per cent. These differences appear to be inherent in the respective mechanisms of analysis and thus point to paper electrophoresis as an independent procedure. The high degree of reproducibility of results and the rapidity and ease of operation experienced with the scanning-recording equipment render it suitable for routine quantitative measurements.

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THE EFFECT OF DIALYSIS OF THE URINE ON UROPEPSIN

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RECENTLY, a paper by Duffin and Kowalewski[1] was published which indicated that dialysis of the urine is necessary for the correct assay of uropepsin. According to these authors, normal and high urinary phenol content cause "serious errors of assay" to the extent that, in the majority of cases, dialyzed urine yielded a considerably higher uropepsin value than nondialyzed urine. As, up to this time, none of the investigators in this field, including this laboratory, has incorporated dialysis in his uropepsin procedure, it was thought desirable to run a series of uropepsin determinations on both dialyzed and nondialyzed urine to ascertain what results would be obtained.

METHOD

The urine specimens were collected under toluene over a twelve-hour period, from 8:00 P.M. to 8:00 A.M., and the subject was instructed to eat nothing and drink nothing but water during this period. The dialyzed urine samples were treated as described by Duffin and Kowalewski.[1] For the nondialyzed urine, a 20 ml. aliquot was adjusted to pH 1.5 and diluted to 25 ml. with distilled water.[2] A 2.5 per cent hemoglobin substrate prepared according to Orringer and associates[3] was employed.* For use, 80 ml. of this substrate was combined with 20 ml. of 0.3 N hydrochloric acid. One milliliter of the adjusted urine was added to 5 ml. of the freshly prepared acidified substrate, previously warmed to 37° C., and the whole incubated at 37° C. for thirty minutes, at which point the enzyme action was stopped by the addition of 10 ml. of 0.3 N trichloroacetic acid. A urinary blank (control) was run for each urine specimen: 10 ml. of 0.3 N trichloroacetic acid was added to 5 ml. of the acidified hemoglobin substrate, followed by 1 ml. of the adjusted urine. The color was developed according to Bucher[4] and read in an Evelyn photoelectric colorimeter using a 540 mμ filter. In making the final calculation, the amount of tyrosine released by the enzyme during the thirty-minute incubation period was divided by three in order to obtain the tyrosine released for a ten-minute period.[2] The errror for this method ranges from 0 to 10 per cent.

RESULTS AND DISCUSSION

Twenty-four men and women and one boy, all normal, participated in this experiment. In Table I, uropepsin values are represented as micrograms of tyrosine released by the enzyme in 1 ml. of undiluted urine during an incubation period of ten minutes at 37° C., in conformance with Duffin and Kowalewski, except that these authors do not state the duration or temperature of incubation. Their incubation period may have been longer than ten minutes, as their concentrations, in general, are much higher than those presented here. The content of chromogenic material of the urine specimens varies over a wide

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*Bovine Hemoglobin Enzyme Substrate Powder, obtained from the Wilson Laboratories, Chicago, Ill., was used.

range and appears to have little effect on the results obtained. The specimens with lower urinary blanks tend to have a lower concentration of uropepsin, which observation is due here to dilution. All our readings fall within the straight line relationship of color vs. tyrosine.

TABLE I. UROPEPSIN AND URINARY BLANK VALUES BEFORE AND AFTER DIALYSIS OF THE URINE

CASE	URINARY BLANK READING (2-LOG G)		UROPEPSIN		URINE VOLUME (ML.)
	NONDIALYZED	DIALYZED	NONDIALYZED	DIALYZED	
1. G. G.	.846	.102	60	60	365
2. L. P.	.745	.140	23	13	225
3. M. B.	.569	.105	98	79	363
4. M. E.	.561	.077	120	92	195
5. C. J.	.549	.081	53	33	259
6. A. F.	.534	.102	152	127	250
7. S. H.	.491	.081	97	78	445
8. J. B.	.472	.097	107	90	266
9. B. P.	.438	.077	13	12	228
10. C. R.	.417	.096	17	20*	350
11. E. E.	.382	.098	97	80	460
12. E. F.	.382	.086	12	10	280
13. B. H.	.369	.072	20	13	588
14. A. S.	.362	.085	120	93	170
15. J. M.	.359	.068	37	40*	615
16. C. K.	.301	.068	10	12*	540
17. G. P.	.292	.077	32	28	606
18. E. S.	.290	.082	50	42	550
19. R. W.	.290	.068	37	25	495
20. A. W.	.288	.092	23	28*	700
21. A. B.	.268	.069	60	57	910
22. R. L.	.266	.069	23	18	530
23. R. T.	.246	.069	19	23*	573
24. S. T.	.189	.068	8	12*	978
25. J. B.	.115	.077	8	13*	800

*Value of the dialyzed sample higher than that of the nondialyzed.

It can be seen from the table, that the uropepsin values for the dialyzed urines tend to be *lower* than those for the nondiayzed samples, in contradiction to the afore-mentioned report. In seven cases, this tendency is reversed with the dialyzed sample, giving a somewhat higher result than the nondialyzed. In all cases, though the difference in uropepsin values between the dialyzed and nondialyzed samples generally exceeds the 10 per cent allowable for error, it is felt that this difference is not significant enough to warrant recommending the incorporation of dialysis into the uropepsin procedure, as the end results and the interpretations drawn from each set of figures would invariably be the same. Mirsky[5] has obtained somewhat similar results and has concluded that dialysis is unnecessary.

SUMMARY

Urine specimens which were dialyzed tended to show *lower* uropepsin values than the nondialyzed samples, in contradiction to a previous report.

It is concluded from the data presented that dialysis of the urine is not necessary for the assay of uropepsin.

I wish to express my appreciation to Dr. H. Necheles for his encouragement during the course of this work.

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THE DETERMINATION OF URINARY ESTROGENS BY FLUORESCENCE

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THE quantitative estimation of urinary estrogens by photofluorimetry is most often performed after heating the sample with sulfuric acid. The experimental conditions have been studied in detail for the pure compounds by Bates and Cohen[1] and the conditions which those authors found best have been applied to urine extracts. Investigations by Goldzieher[3] and Slaunwhite and associates[8] have established that the intensity of fluorescence is greatest for the natural estrogens at about 485 mμ. The absorption spectra for sulfuric acid-treated samples of the same compounds show maxima at about 455 mμ.[2, 3] An adaptation of the Beckman Model DU Spectrophotometer has been developed in this laboratory.[7] With this instrument the sulfuric acid-treated estrogens are irradiated with light of a wave length approximately that of the absorption maxima. The fluorescent output measured is that at the fluorescent maximum. It is felt that in this way the effects of interfering compounds in urinary extracts are reduced to a minimum. Internal standards were included in each sample to reduce the error caused by quenching effects of compounds present in urine extracts, and also to correct for differences in self-absorption of fluorescent light by different solutions.[4]

It has been observed[1] that NO_3^- is very effective in destroying fluorescence of estrogens in sulfuric acid. This observation was employed in this series of experiments to correct for nonspecific fluorescence. The fluorescence of the sample solution was determined, one drop of a saturated solution of potassium nitrate was added, and the fluorescence was remeasured. The difference in fluorescence was assumed to have been due to estrone, and each solution thus served as its own reagent blank.

EXPERIMENTAL

Extraction of Estrogenic Substances.—Morning urine samples were collected daily from three normal women throughout one menstrual cycle, and from two men for eight successive days. The time in hours which the sample represented was noted, and excretion on a twenty-four-hour basis was calculated. It is recognized that excretion probably varies on a diurnal pattern, but it was felt that the conditions of urine collection were sufficiently uniform that such a calculation was valid. A 250 ml. aliquot of the sample was acidified with 15 volumes per cent of concentrated hydrochloric acid (if the sample was less than 250 ml., the entire sample was used). The acidified urine was refluxed for thirty minutes and cooled in an ice bath.

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The hydrolyzed sample was extracted with 5 × 50 ml. of ether freshly distilled over $FeSO_4$ in 1 N H_2SO_4. The combined ether extracts were washed with 50 ml. of distilled water. A second washing with 15 ml. of distilled water was often necessary if an emulsion had been present. The ether extract was then washed successively with 2 × 25 ml. of a saturated solution of sodium bicarbonate and 25 ml. of distilled water. The estrogens were extracted with 4 × 50 ml. of 10 per cent KOH and the ether solution was discarded.

The potassium hydroxide extracts were combined and the pH was adjusted to 9 ± 0.5 with Du Pont sulfuric acid. The estrogens were extracted with 3 × 65 ml. portions of peroxide-free ether, and the combined ether extracts were washed with 3 × 40 ml. of distilled water. The ether extract was filtered through anhydrous Na_2SO_4, evaporated to dryness under reduced pressure (at a temperature below 50° C.), and the residue was taken up in 3 ml. of redistilled ethanol. The samples were stored at -25° C. until analyses were performed.

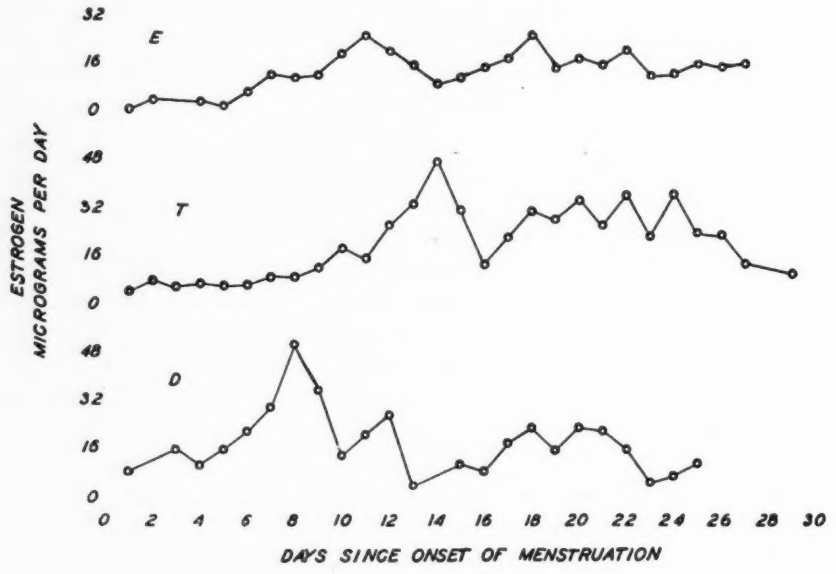

Fig. 1.

Development of Fluorescence.—Fluorescence was developed and readings were made in Klett colorimeter tubes. The tubes were prepared as follows:

Sample—0.1 ml. ethanol + 0.1 ml. ethanolic solution of sample.

Sample + standard—0.1 ml. ethanol containing 0.166 γ estrone + 0.1 ml. ethanolic solution of sample.

To each tube was added 1 ml. of a 90 per cent by volume solution of H_2SO_4 (Du Pont). The tubes were heated for ten minutes at 80° C. (± 5°) and cooled in a water bath. Six milliliters of a 65 per cent by volume solution of H_2SO_4 (Du Pont) were added and the fluorescence of the samples was measured.

Measurement of Fluorescence.—Fluorescence was measured on a modification of the Beckman Model DU Spectrophotometer developed in this laboratory.[7] No standards for control are necessary since each sample is measured against its own standard. The slit width was always 0.3 mm., and the sensitivity was set so that readings were possible on each sample. The instrument was used on the 0.1 scale setting. Exciting light was that transmitted from an incandescent bulb by a Bausch & Lomb interference filter with a

maximum transmission at 455 mμ of 41 per cent, and a half-width of 9 mμ. Measurements of fluorescent light were made at 480 mμ. Fluorescence intensity was read from the transmitting scale.

After the fluorescence of the sample and the sample plus standard had been read, the fluorescence in the sample tube was destroyed by the addition of one drop of a saturated solution of KNO_3. Previous experiments had indicated that this quantity of the oxidizing agent was sufficient to destroy the fluorescence of 0.17 γ of estriol or 0.58 γ of estrone. The fluorescence was determined again and this value was taken to represent the fluorescence of the solution caused by substances other than estrogens.

Calculations.—The difference in readings between the sample and the sample plus standard gives a number which represents the intensity of fluorescence for a standard quantity of estrone. The difference between the reading for the sample alone before and after treatment with KNO_3 gives a value representing the intensity of fluorescence for an unknown quantity of estrone. A simple proportion then serves to calculate the quantity of estrogen in the unknown sample.

RESULTS AND DISCUSSION

The results obtained from the determinations are shown in Fig. 1 for women and in Table I for men. The curves for the women have the typical peaks which have been established by many investigators.[5, 6] The values shown for the men are lower than those usually obtained, but this may be because a portion of the interference of fluorogenic, nonestrogenic substances[8] has been eliminated. The important observation, to us, is that positive results have been obtained for each sample. This has not always been the case in the experience of one of us with another fluorescence meter. (McAnally, unpublished data). The urine extracts which gave the results plotted in Fig. 1 as *E* were read in a Coleman Photofluorimeter by using a 435 mμ and a 360 mμ lamp filter in conjunction with a PC-9A phototube filter. In all but one case, the reading with the 360 mμ filter was larger than that with the 435 mμ filter, and calculations were impossible.

TABLE I. MICROGRAMS OF ESTROGENS EXCRETED PER TWENTY-FOUR HOURS IN URINE OF TWO MALES

	SUBJECT	
	1	2
First day	8.1	17.0
Second day	7.0	8.7
Third day	8.1	15.0
Fourth day	5.4	15.0
Fifth day	2.7	11.4
Sixth day	9.4	12.0
Seventh day	12.4	14.3
Eighth day	9.9	9.4

It is realized that this method has not removed all of the debatable points from this determination. The effects of mixing estrone and estriol are not accounted for and the treatment with potassium nitrate cannot be assumed to destroy only fluorescence due to estrogenic substances. It is felt that the method is an improvement over those which do not take into account self-absorption or quenching effects, and that a greater degree of accuracy is effected by making some effort to correct for nonestrogenic fluorescence.

SUMMARY

An improved method for the estimation of urinary estrogens is outlined, together with results obtained on both male and female subjects.

We wish to express our indebtedness to Dr. R. T. Hill for his counsel and assistance in the carrying out of this project.

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A SIMPLE METHOD FOR THE QUANTITATIVE DETERMINATION OF ALPHA AND BETA LIPOPROTEINS IN SERUM BY PAPER ELECTROPHORESIS*

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IN THE study of atherosclerosis, the serum lipoprotein fractions are of importance. Gofman[1] using the ultracentrifuge, and Barr[2] using the chemical fractionation technique of Cohn,[3] have succeeded in demonstrating differences between normals and atherosclerotics. Since the afore-mentioned methods require expensive apparatus or complex techniques, a simpler procedure is desirable. The serum lipoproteins are adaptable to a simple quantitative electrophoretic procedure using filter paper. It is the purpose of this paper to present such a method.

Strips of Schleicher and Schull No. 2043B filter paper were moistened in a Veronal buffer, at pH 8.55 and ionic strength 0.125. They were then streaked with 0.01 ml. and 0.04 ml. serum and run simultaneously for twenty-four hours at 4 MA. The strips of filter paper were then dried in air. The protein pattern was visualized by staining the strips streaked with 0.01 ml. serum with a saturated solution of Amido Black 10-B in 4.5 parts of methanol, 1 part of glacial acetic acid, and 4.5 parts of water for five to ten minutes. The excess stain was washed off with this same methanol-acetic acid-water solution and the paper strips dried in air. The lipoproteins were visualized by straining the strips streaked with 0.04 ml. of serum, in a saturated solution of Sudan III in 4.0 parts of ethanol, 1 part of acetone, and 4.0 parts of water for twenty-four hours, and removing the excess stain with the ethanol-acetone-water solution. The paper strips were then dried in air.

After using the saturated solution of dye for staining, it should be recombined with the saturated stock solution containing excess dye. Before using again, the stock solution should be filtered. If a filtered saturated solution is used over a period of time, the stain becomes progressively weaker and the patterns become less intense.

Figs. 1 and 2 are typical curves obtained in a normal case and in a case of atherosclerosis, respectively. The curves were obtained by plotting the density of the spots measured by means of a densitometer, against the distance migrated from the point of application. Since pure samples of alpha and beta lipoproteins were not available as standards, the results are arbitrarily expressed as square inches of area under the plotted curve. The area was meas-

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TABLE I. ALPHA AND BETA LIPOPROTEINS IN NORMAL INDIVIDUALS

MALE			FEMALE		
AGE	ALPHA LIPOPROTEINS (IN.² 0.04 ML.)	BETA LIPOPROTEINS (IN.² 0.04 ML.)	AGE	ALPHA LIPOPROTEINS (IN.² 0.04 ML.)	BETA LIPOPROTEINS (IN.² 0.04 ML.)
	0.55	2.13		0.68	1.63
	0.94	1.91		1.23	1.30
	1.42	2.23		1.00	1.59
20-29	1.20	2.42	20-29	0.75	1.98
	1.21	2.30			
	0.66	1.99		1.21	2.12
	1.08	2.14		1.19	1.35
	1.18	2.09			
	1.04	1.68		1.23	1.71
	1.09	1.51		1.43	2.14
30-39	1.44	1.78	30-39	1.32	1.57
	0.93	2.07		1.06	1.54
				1.21	1.46
	1.63	1.86			
	1.28	1.93			
	0.71	2.40		1.14	2.14
	1.13	2.20	40-49	1.11	1.71
				1.07	1.57
40-49	1.01	1.91		0.97	2.03
				1.06	2.15
	1.46	2.31		1.03	1.72
				0.98	1.85
	1.13	2.16		0.91	1.58
	1.19	2.33			
	1.17	1.87		1.33	2.10
				1.26	2.32
50-55	1.12	2.33	50-55	1.57	1.86
	1.11	2.06			
	0.95	1.93			
	0.97	1.56			

The concentrations are expressed as square inches of area under the plotted curves of 0.04 ml. of serum.

ured by a planimeter calibrated in inches. The serum liproprotein concentrations are therefore expressed as square inches per 0.04 ml. serum. At present, values expressed in this manner are being compiled on a group of normal men and women from 20 to 55 years of age. This series, not yet completed, is listed in Table I. These values indicate that the beta lipoproteins tend to run higher in men in their twenties. In order to test the reproducibility of the method, some of the determinations were run in duplicate. Wherever this has been done, duplicate results are listed. Good agreement is observed in most cases.

The purpose of the protein patterns is to fix the position of the lipoprotein fractions. Figs. 1 and 2 indicate that the peak of the alpha fraction lies between the alpha-1 globulin and the albumin, while the beta fraction lies between the gamma and beta globulins. Once the position of the lipoproteins in relation to the proteins has been fixed, a protein separation pattern need not be run with each lipoprotein determination, unless the presence of abnormal lipoproteins is suspected. It can be demonstrated that the migration rates of

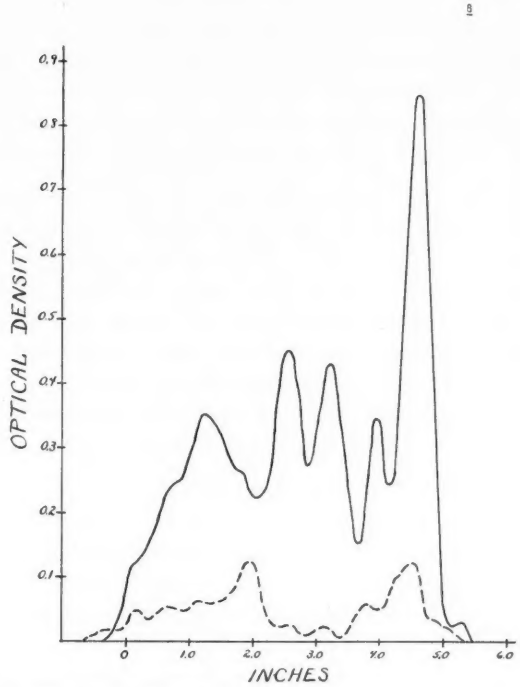

Fig. 1.—Serum protein and lipoprotein fractionation curves in a normal case. A solid line denotes the protein curve. A broken line denotes the lipoprotein curve.

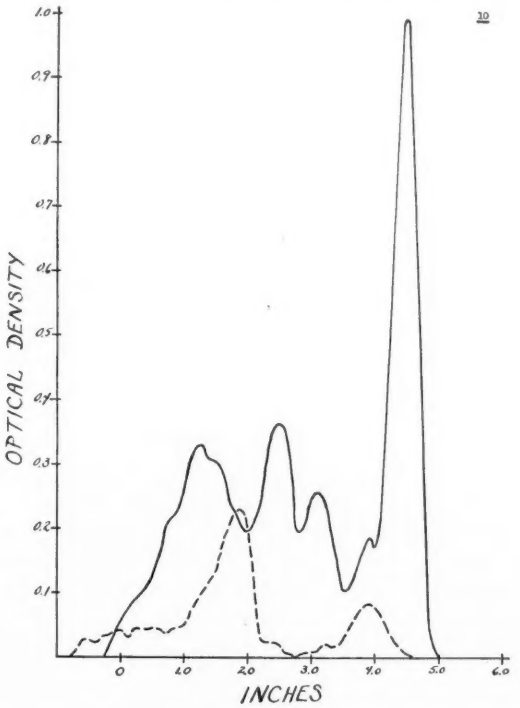

Fig. 2.—Serum protein and lipoprotein fractionation curves in a case of atherosclerosis. A solid line denotes the protein curve. A broken line denotes the lipoprotein curve.

the lipoproteins are identical with 0.01 and 0.04 ml. serum. Therefore, the lipoprotein pattern obtained with 0.04 ml. serum can be compared with the protein pattern obtained with 0.01 ml. serum.

One point must be stressed. Different lots of Sudan III will stain with varying intensities, even though obtained from the same company (National Aniline Division of the Allied Chemical and Dye Corporation). Consequently, the values here listed will apply only to that batch of Sudan III labeled "Lot 13" which was used in this study. Investigators using different lots of the dye will obtain curves differing in area from the ones in this investigation. However, the following observation has been made. If a lipoprotein pattern is first obtained with a solution giving a less intense stain and then is restained in a saturated solution yielding a more intense stain, the lipoprotein pattern will acquire the same intensity as if it were directly stained by the more intense solution.

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A NEW METHOD FOR THE RAPID IDENTIFICATION OF DERMATOPHYTES

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FOR many years the standard method for the isolation and identification of the dermatophytes has been the use of Sabouraud dextrose agar. Potato dextrose agar and cornmeal agar with dextrose have been suggested for differentiating between *Trichophyton mentagrophytes* and *T. rubrum* on the basis of pigment formation.[1] In recent years several mycological culture media have been introduced, among them Littman oxgall agar.[2]

While these media are generally satisfactory, they nevertheless have several distinct disadvantages:

1. The fungi grow slowly and produce their characteristic pigments after long periods of time. Several weeks are sometimes required before the identity of the fungus is definitely established. Furthermore, the pigments are not clearly defined.

2. The agar shrinks, due to syneresis, and the discoloration of the media on prolonged storage may introduce some difficulty in observing the pigmentation on the reverse side of the colony.

3. The culture media containing antibiotics, namely Littman oxgall agar and Sabouraud dextrose agar enriched with penicillin and streptomycin, are unstable and must be prepared every few weeks in spite of refrigeration.

Sand, glass powder, paper, gauze, etc., impregnated with nutrient material, have been suggested as a substitute for agar in promoting bacterial growth.[3] Glass fabric[4] and wool fibers[5] have also been suggested as absorbents for mycological culture media. Aside from solving the problem of syneresis, the basic problems mentioned previously still remained unsolved.

The purpose of this paper is to describe several innovations in the cultivation and identification of fungi, resulting in a more stable culture upon which the yeasts and molds will grow profusely and will produce their characteristic pigments, if any, more rapidly and more distinctly.

MATERIALS AND METHODS

A large sheet of highly porous and thick filter paper (S. & S.* No. 725) is dyed black with aniline black, washed with a detergent, and finally rinsed with water until the paper is entirely free from any soluble dye and detergent. (Whatman No. 29 black filter paper has been used satisfactorily, although the paper is not as thick as is desired.) This black paper is then painted on one side with a 10 per cent aqueous suspension of calcium carbonate, containing 0.5 per cent gum arabic, and is then permitted to dry. A similar undyed sheet is then placed on the black sheet in such a manner that the chalk is

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sandwiched between the black and white papers. The two sheets are then stitched together on a sewing machine in such a manner that when the papers are finally cut into strips, 1¼ by 1¾ inches, the stitches will appear on the edge of each strip along the two longer dimensions only. Each strip is then inserted into a vial, 28 by 57 mm., in a curved position with the black surface on the concave side.

About 3 ml. of a nutrient material is then added to the vial, the cap loosely replaced, and the whole unit is then sterilized at 10 pounds for 15 minutes. The vial is cooled while lying in a horizontal position and the cap tightened, when cold, to prevent evaporation.

The nutrient liquid may be any one of the standard mycological media now in use in the form of agar slants or in the form of broths. I, however, found the following formula very effective in producing an early luxuriant growth plus earlier pigment production:

Peptone	10 Gm.
Glucose	40 Gm.
Potassium acid phosphate	1 Gm.
Thiamin hydrochloride	5 mg.
Tween 80 (sorbitan mono-oleate)	8 ml.
Agar	10 Gm.
Water	1 L.

DISCUSSION AND RESULTS

The black surface is inoculated in the usual manner with the exudate or scrapings of the lesion involved and the cap is loosely replaced. Since this black surface, unlike an agar slant, is firm and rigid, the transferred material may be pressed more firmly against the paper. A growth will be perceptible in the shortest possible time since the jet-black surface greatly aids in making the yeast and fungus colonies readily visible almost as soon as they are formed. The characteristic pigments, if any, will be readily visible on the reverse white surface (see Fig. 1 and Table I).

TABLE I. COMPARISON OF GROWTH AND OF PIGMENT PRODUCTION OF THE FUNGI RESPONSIBLE FOR DERMATOPHYTOSIS OF THE FEET (AVERAGE DAYS)

ORGANISM	EARLIEST PERCEPTIBLE APPEARANCE OF GROWTH		EARLIEST APPEARANCE OF A SIZEABLE* CHARACTERISTIC COLONY		EARLIEST APPEARANCE OF A CHARACTERISTIC PIGMENT	
	SABOURAUD'S AGAR	IMPROVED METHOD	SABOURAUD'S AGAR	IMPROVED METHOD	SABOURAUD'S AGAR	IMPROVED METHOD
T. rubrum	5 to 6	3 to 4	8 to 10	6 to 8	12 to 14 (red)	7 to 8 (red)
T. mentagrophytes	4 to 5	3 to 4	6 to 8	4 to 6	inconclusive‡	5 to 7 (yellow)
E. floccosum	6 to 8	3 to 4	8 to 10	6 to 8	15 to 18	8 to 10 (greenish yellow†)
C. albicans	1 to 2	1 to 2	4 to 5	4 to 5	—	—

*About 1 cm. in diameter.

†On face of colony in addition to the pigment on reverse side.

‡On Sabouraud dextrose agar *T. mentagraphytes* produces a pigment on the reverse side varying from yellow to brown to pinkish-tan to colorless.

The Tween, a nonionic surface active agent, not only aids in the more efficient diffusion of the pigment, but also seems to be directly responsible for an earlier production of pigment. This last property of the Tween may be due to its ability to dissolve the waste products and toxic material and prevent

their accumulation at the surface,[6] or it may possibly be due to traces of free oleic acid in the ester. While a higher concentration of Tween will cause some fungi to produce their pigments very early (at a concentration of 5 per cent Tween, *T. rubrum* will produce an intense red pigment in about five to six days), a concentration above 0.8 per cent is undesirable because it will inhibit the growth of such yeasts as *C. albicans*.

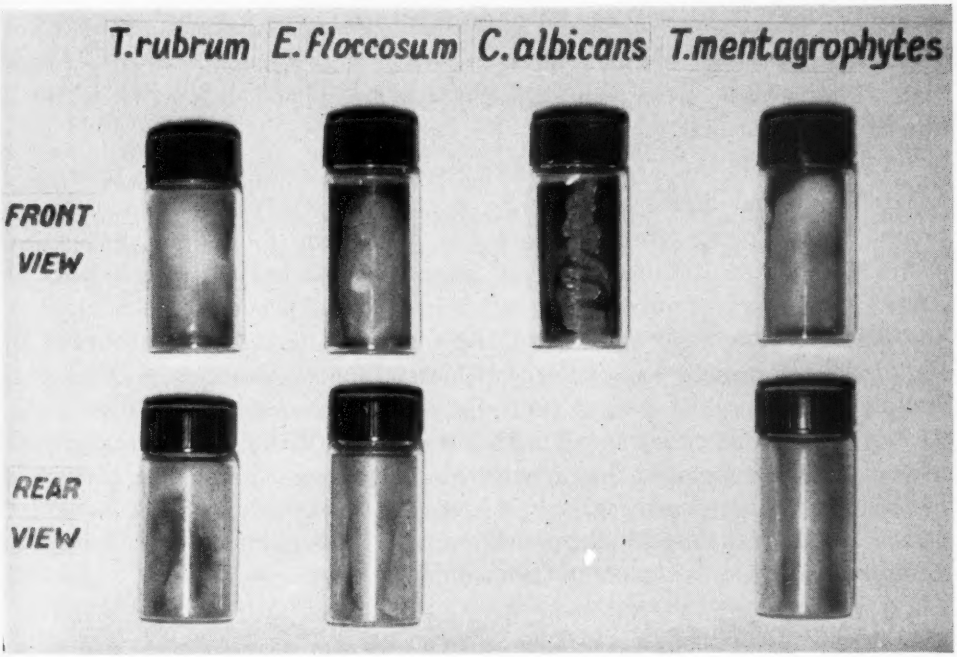

Fig. 1.—Cultures of fungi responsible for dermatophytosis of the feet (front and rear views).

The function of the calcium carbonate between the black and white papers is to intensify the colors of the pigments, if any, that are produced by the fungi. It has been shown that many of the pigments produced by the dermatophytes are chemical indicators[7]; *T. rubrum,* for example, will turn from yellow to red to purple as the pH of the medium rises. As the pigment diffuses away from the black paper, it passes through the layer of calcium carbonate and is neutralized by the chalk, the indicator being in its acid form at the pH of the medium (about 5.5). The calcium carbonate, therefore, intensifies the color of the pigment almost as soon as it is formed in the mycelial mat of the colony. These color changes ordinarily take place very slowly on the reverse side of fungus colonies growing on standard agar slants and plates, due to the fact that the peptone in the media, by virtue of its buffer action, will resist changes in pH to alkalinity in a short period of time, an alkalinity that must exist in order for the pigments to be readily perceptible.

It has, furthermore, been shown that many of these pigments assume one color in reduced form and another in an oxidized form,[8] the colors being more intense in the oxidized state. In the standard agar slants and plates it is undoubtedly the dissolved oxygen that is responsible for the final color of the pigments. With this improved method, most of the white paper, representing the reverse pigmented side of the colony, is directly exposed to the atmosphere of the vial and thus the pigment possibly reaches a higher state of oxidation more readily. It may be noted that when the reverse side of *T. rubrum* is examined, the white paper is highly colored with an intense red pigment in those areas where the white paper does not adhere firmly to the vial, while in those areas where the paper is not immediately exposed to the atmosphere of the vial, the pigment does not readily reveal itself.

The Tween in the medium plus the porous nature of the black paper will restrict to some degree the growth of many of the saprophytes and contaminants that very often overgrow the pathogenic colonies on Sabouraud slants and plates. (Littman oxgall agar accomplishes this localization of contaminants very effectively but, as has been stated previously, it is unstable and colonies grow even more slowly on that medium than on Sabouraud's). Restriction of growth was noticed with such contaminants as *A. fumigatus* and Penicillium, while with *A. niger* no localization was noticed. Actidione, 0.5 mg. per milliliter may be added for a more effective restriction of growth but only at the expense of the early growth and pigment-production properties of the medium. The growth of bacterial contaminants is inhibited by virtue of the low pH (about 5.5) of the medium and by the action of the Tween, in addition to the porous surface of the medium.

This new method, aside from finding application in the rapid identification of the dermatophytes, may be useful for other purposes:

1. In studying the pigments produced by fungi, the white paper containing the pigment may be conveniently separated from the black paper containing the colonies in order to simplify the extraction of the pigment. The calcium carbonate may be omitted for that study and larger discs of filter paper may be used and placed in Erlenmeyer or Kolle flasks. Various reagents may also be readily dropped on the white paper for spot tests without previous extraction of the pigment.

2. Other insoluble material may be substituted for the calcium carbonate layer for suitable chemical combinations and color reactions.

3. The impregnated sterile paper may be dried in a desiccator and kept in this dehydrated form for indefinite periods of time. For use, one simply adds a premeasured amount of sterile water to the vial (a medicine dropper full of boiled water). The vial is then kept in a horizontal position for about thirty minutes when a homogeneous gel is formed once again within the pores of the paper. This simple process of preparing a culture medium from its dehydrated form can only be accomplished when all the solids have previously been absorbed by the highly porous paper, since here the dried material goes

into solution without heating. This should prove to be a great convenience in a small laboratory, the office, and in the field where autoclaves are not readily available.

4. The whole element may be transferred from the vial to the stage of a microscope, at any stage of development, for close examination with reflected light without disturbing the mycelia of the colony. This is of considerable value in morphologic studies and as a general teaching aid.

5. I have found this method very satisfactory in preparing preserved specimens of the various filamentous fungi for teaching and record purposes. After the fungus colony has reached the desired size and color, the cap is removed and the vial placed in a desiccator for several days. The vial is then removed from the desiccator, exposed to the fumes of formaldehyde for several hours, and the cap is replaced. The aerial mycelia seem to retain their forms almost indefinitely and the pigment, if any, on the reverse side remains bright and intense when protected from light. The yeast cultures are best preserved by adding two drops of formalin to the vial and keeping the contents moist by tightening the cap.

SUMMARY

An improved method for the isolation and identification of the dermatophytes, with special reference to the ones responsible for dermatophytosis of the feet, is described. It involves the addition of a nutrient material to an element composed of porous strips of filter paper with calcium carbonate in between. The jet-black surface, upon which the inoculation is made, will detect the earliest possible growth of the fungus or yeast, and the calcium carbonate will intensify the color of the pigment that may be produced, causing the color to be readily perceptible on the reverse white paper.

The formula for a nutrient material enriched with thiamin, phosphates, and Tween is given. Colonies appear and produce their pigments in about two-thirds of the time required on Sabouraud's medium. The pigments are more intense and are more clearly defined. This formula may also find application as an agar slant or plate.

Other possible advantages of this method are: (1) simplicity in the isolation and extraction of mold pigments; (2) convenience in microscopic examination of cultures; (3) its preparation in the form of a dehydrated culture medium that may be hydrated for use by the addition of cold sterile water; and (4) a convenient method for preserving cultures for teaching and record purposes.

I wish to express my appreciation to Vera B. Dolgopol, M.D., Andrew H. and Royal M. Montgomery, M.D., and to Izaak Dworecki, M.D., who have read the manuscript and have made many valuable contributions.

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A SIMPLE DEVICE FOR THE RAPID EVAPORATION OF CHROMATOGRAPHIC SAMPLES

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PARTITION chromatography with liquid chromatograms requires the analysis of a large number of fractions. Since evaporation of the fractions usually is a tedious procedure, it was considered desirable to develop a method to facilitate the removal of solvents. The simple device described here achieves this purpose by the simultaneous heating and vigorous blowing of the samples; it has proved to be satisfactory for organic solvents with a boiling point between 40° and 160° C. Its great advantage is the considerable curtailment of the evaporation time, e.g., ten samples each containing 50 ml. of ethanol may be completely evaporated in seven to eight minutes.

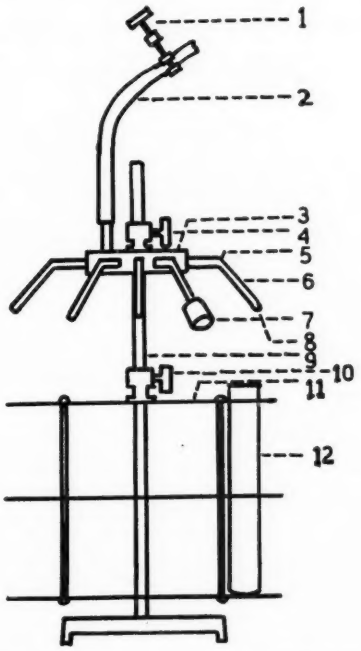

Fig. 1.—Diagram of the apparatus. For explanation, see text.

The essential features of the apparatus (see Fig. 1) are a pipe system (*3*) serving as the air-supply and a test-tube rack (*11*); both are removable and are attached to a stand (*9*) by means of screws (*4, 10*). The rack is made for ten Pyrex test tubes, 200 by 26 mm., in which the fractions are collected.

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The pipe system (*3*) ensures a sufficient supply of compressed air to the test tubes (*12*) to keep the solvent in vigorous circulation. The maximal effect is obtained when the air outlets (*6*) are kept as close as possible to the wall of the test tubes, and are about 3 to 5 mm. below the edge of the tubes. When only a few samples are to be evaporated, the superfluous outlets should be plugged (*7*).

The compressed air is taken from a compressor or a gas-tube, which is connected to the air-supply system through a vacuum rubber tube (*2*). The rate of flow is regulated by means of a clamp (*1*). It has been found advantageous to begin with relatively low pressure and to increase it gradually. Excessive pressure may result in splashing. On the other hand, the greater the pressure, the more rapid is evaporation, and the less is the risk of overboiling and self-ignition.

During evaporation, the apparatus is placed in an electrically-heated water bath in a hood with good ventilation. The immersion of the test tubes in the boiling water bath should be adjusted to the quantity and the nature of the solvent to be evaporated. For example, tubes containing 10 to 15 ml. of solvents with a low boiling point may be completely immersed; this also applies to larger quantities of solvents with a higher boiling point. Larger quantities of solvents with a low boiling point, e.g., ether or petroleum ether, must not be immersed deeper than 2 to 3 cm.

The apparatus may be constructed of galvanized copper or of other material which withstands organic solvents. To obtain the maximal effect, the angle of the outlets (*5*) should be about 130° to 150°, the hole (*8*) through which the compressed air is ejected, about 1.0 to 1.2 mm., and the pressure about 0.4 to 0.6 kg. per square centimeter per outlet.

Erratum

In the article by King, Ward, and Raney, entitled "Two Simple Media for the Determination of Pyocyanin and Fluorescin," which appeared in the August, 1954, issue of the JOURNAL, pages 301-307, beginning with the third sentence under "Summary" on page 307 the remainder of the paragraph should read as follows: "One hundred (93 per cent) of the 107 strains of Pseudomonas studied were detected by pigment production on medium B; all 107 strains could be detected by the combined use of media A and B. On Difco proteose peptone No. 3 agar 77 per cent of the same strains elaborated a water-soluble pigment, 40 per cent were positive on Difco heart infusion agar and 27 per cent on tryptose agar. The reactions produced on the two media by organisms belonging to certain other genera are described."